*Year :* 2017

# The loss of self-incompatibility and the évolution of mixed mating in the perennial herb Linaria cavanillesii

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THÈSE DE DOCTORAT

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

pour l'obtention du grade de

Docteur ès sciences de la vie

par

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Jury

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LAUSANNE
2017

La Repro
Université de Lausanne
2017

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

**Ecole Doctorale**

**Doctorat ès sciences de la vie**

# Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

| | | |
|---|---|---|
| ***Président·e*** | Monsieur | Prof. Ron **Stoop** |
| ***Directeur·rice de thèse*** | Monsieur | Prof. John **Pannell** |
| ***Experts·es*** | Madame | Prof. Tanja **Schwander** |
| | Monsieur | Dr Pierre-Olivier **Cheptou** |

le Conseil de Faculté autorise l'impression de la thèse de

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Master de l' Université de Lausanne

intitulée

**The loss of self-incompatibility and the evolution of mixed mating in the perennial herb *Linaria cavanillesii***

Lausanne, le 7 avril 2017

pour le Doyen
de la Faculté de biologie et de médecine

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et de médecine
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Lausanne - 2017

# REMERCIEMENTS

I would like to give special thanks to John, who supervised me successfully through the years. I remember when I first met him at the Evolutionary Biology Workshop in Arolla, one of the beautiful places in Switzerland. I was already amazed by John's kindness and spontaneous nature, and this first impression definitely influenced my decision to start a PhD with him. Throughout the years, I have always appreciated his unwavering support. He also successfully brought my fascination for plants to the next level, which is something I am really glad of! Finally, I could never thank him enough for the time and effort he invested during the last months before my thesis submission. While I am writing these words, he is probably still working hard to help me get finished…

Je n'aurais jamais été capable d'accomplir cette thèse sans l'aide précieuse de nombreux étudiants et collaborateurs de Lausanne : Dessislava Savova Bianchi, Rachid (l'universel), Loïc Liberati, Melvyn Staiff, Sébastien Tesson, Ana Paula Machado, Camille Roux, Quentin Rougemont et Yves Cuenot. Mais aussi sur le terrain en Espagne : John, Nathalie, Rachid et mon père. Je tiens aussi à remercier Nathalie Jollien et Samuele Testa, avec qui j'ai partagé mes premières expériences de supervision de Master! Finalement, je souhaiterais remercier tout particulièrement Nicolas Ruch. Mis à part sa nature amicale à tout épreuve, il a aussi toujours été d'un support permanent et s'est occupé de mes plantes comme personne.

Je tiens à (presque) remercier ceux qui ont failli me faire rater ma thèse. Si la qualité de celle-ci s'en ressent, c'est leur faute ! Alors au nom des bars perpendiculaires (!), des tarots, des pokers (FIPE), des ciné-club, des apéros en tout genre, des défis sportifs, des pétanques, des discussions de révolutions écolos, des randos, de la musique, de tout, MERCI ! A tous ceux qui se reconnaissent aujourd'hui, et en particulier aux pionniers : Alex, Auré, Céline, Geoff, Gordon, Guillaume, Laura, Zoé et aux masters BEC 2011.

A Geoffrey (alias Jazzy Jeff), parce que nos pas partagés nous ont menés jusqu'ici aujourd'hui. Pour le meilleur de toutes ces années passées ensemble: la complicité, le partage, le rire, l'amour, la simplicité, la découverte et tant d'autres choses encore inaltérables. A sa famille aussi, avec qui je garde de nombreux souvenirs joyeux.

A ceux qui compteront toujours, tout particulièrement mes parents, Yoann, Nicolas et Louis; pour tout simplement être une famille qui s'aime. Je n'oublie pas les pièces rapportées sans qui nous serions surement un peu moins heureux (Dan, Hannah et Camille), et une pensée particulière pour Aurélie.

Alors à la passion; à tout ce qui fait qu'on découvre, qu'on vibre, qu'on aime et qu'on se sent vivant!

# ABSTRACT

Understanding contrasting strategies developed by organisms can at first sight be puzzling, but they are often a good example of how diversity arises: one strategy may be advantageous in certain circumstances, but not in others. One of such example is between hermaphrodite individuals that reproduce via outcrossing, and those that, in certain conditions, shift to self-fertilization, e.g., after the breakdown of a genetic self-incompatibility mechanism. Transitions from self-incompatibility (SI) to self-compatibility (SC) are common in flowering plants, and the focus of this PhD thesis is to understand what forces can help to constrain or promote these shifts. Importantly, after the loss of an SI mechanism, plants can evolve towards complete selfing (all their ovules are fertilized by their own pollen), maintain outcrossing by other means (their ovules are fertilized by other mates' pollen), or they may show mixed mating (seeds results from both selfing and outcrossing events). Mixed mating is more common in SC plants than previously thought, but how mixed mating can be maintained remains a puzzle. In this thesis, I attempt to answer questions about the causes and consequences of SC *vs.* SI in plants, as well as variation in mating systems that ensue. In particular, I attempt to understand what forces may help to explain transitions from SI to SC, and the dynamics of consequences of this shift once the transition has occurred. For instance, how fast can morphological or genetic changes occur after a recent transition to SC? Do newly evolved SC populations show patterns of reduced genetic diversity, increased population differentiation or reduced flower morphology, as expected by theory? What forces might maintain mixed mating in populations that have shifted to SC? And, finally, how fast can SC spread in other populations once it has been fixed in part of a species' range? To address these questions, I use the perennial Spanish herb *Linaria cavanillesii* as a model, a species that presents discrete variation in compatibility phenotypes among populations. In particular, *L. cavanillesii* possesses strict outcrossing SI populations, outcrossing populations with 'leaky' SI, and a recently derived SC population showing mixed mating; it thus offers ideal material with which to test questions about the evolutionary consequences of a recent shift to SC. During the thesis, I found some patterns that are consistent with theory and empirical observations from other species (e.g., a reduced genetic diversity in the SC population and strong inbreeding depression in the SI populations). However, some other observations were more puzzling, such as the absence of a selfing syndrome, and inbreeding depression in the recently derived mixed mating SC population where plants are nevertheless able to self in the absence of pollinators. My experiments also indicate that SC confers increased fitness and can spread remarkably rapidly while in competition with SI, raising the question as to why we may still observe the maintenance of both SC and SI in *L. cavanillesii*. I discuss the results of my research in terms of the interesting questions it raises about the reproductive biology of *L. cavanillesii* in particular, and the light it throws on our understanding of plant mating-system evolution more generally.

# RESUMÉ FRANÇAIS

Que des organismes apparemment similaires puissent développer des stratégies contrastées peut de prime abord laisser perplexe, mais cette variation est souvent un très bon exemple de l'émergence de la diversité: une stratégie peut être avantageuse dans certaines circonstances, et non dans d'autres. Par exemple, certains individus hermaphrodites se reproduisent avec d'autres individus (fécondation croisée) et évitent ainsi les effets néfastes de la consanguinité, mais dans certaines situations, il peut au contraire être bénéfique de se reproduire par autofécondation après l'arrêt du fonctionnement des mécanismes génétiques d'auto-incompatibilité. Les transitions d'un système d'auto-incompatibilité (AI) à un système d'auto-compatibilité (AC) sont très répandues chez les plantes, et le but de cette thèse est de comprendre quelles forces vont contraindre ou au contraire promouvoir le passage de l'un à l'autre. De plus, après la transition vers l'AC, les plantes peuvent soit passer à une fertilisation auto-fécondante totale (tous les ovules sont fertilisés par le propre pollen de la plante), soit maintenir une fécondation croisée (les ovules sont fertilisés par le pollen d'autres plantes) ou un mélange des deux (certaines graines résultent d'autofécondation et d'autres de fécondation croisée). Cette « fécondation mixte » est incroyablement commune chez les plantes AC, mais ce n'est pas toujours évident d'identifier si ces populations sont stables. Dans cette thèse, j'essaie donc de comprendre les causes et conséquences du polymorphisme AI vs AC chez les plantes à fleurs. En particulier, j'essaie d'identifier les forces importantes qui peuvent influencer la transition de AI à AC, ainsi que la dynamique des conséquences une fois que la transition a eu lieu. Par exemple, à quelle vitesse peut-on observer des changements morphologiques ou génétiques après une transition récente à l'AC ? Quelles forces maintiennent la « fécondation mixte » ? Et finalement, avec quelle rapidité l'AC, une fois fixée, peut-elle se répandre dans d'autres populations, et donc pourquoi observe-t-on toujours du polymorphisme AC vs AI au sein de la même espèce ? Pour répondre à ces questions, j'utilise la plante *Linaria cavanillesii*, une espèce récemment décrite et endémique du sud-est de l'Espagne. Cette espèce présente une variation intéressante dans ses systèmes de reproduction entre populations et offre l'opportunité de tester des questions reliées aux conséquences évolutives d'une transition récente vers l'AC. Certaines observations sont en accord avec la théorie et d'autres études empiriques (par exemple une réduction dans la diversité génétique chez la population AC, ou une forte dépression de consanguinité dans les populations AI). Cependant, d'autres observations sont inattendues, comme l'absence de changements morphologiques de la fleur ou de dépression de consanguinité chez la récemment divergente population AC. J'ai aussi trouvé que lorsqu'elles sont en compétition avec des plantes AI, l'AC peut se répandre très rapidement, et je discute donc des conséquences de ce résultat pour *L. cavanillesii*, mais aussi pour la compréhension des systèmes de reproduction chez les plantes plus généralement.

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# - GENERAL INTRODUCTION -

# *GENERAL INTRODUCTION*

When one starts paying attention to the plant world that surrounds us, one cannot help but notice the wide diversity of strategies deployed to cope with the main challenges of every living organism: surviving and reproducing. Of course, it begins to make sense when we realize that plants cannot move but still have to cope with various pressures imposed by their environment. These selective pressures lead species to diverse evolutionary strategies. A classical example of contrasted strategies that have been studied for decades is between flowerings plants (angiosperms) that are self-incompatible (SI) obligate outcrossers and self-compatible (SC) plants that are able to self-fertilize. SI is though to be ancestral in many families of plants (Allen and Hiscock, 2008), but the breakdown of SI has been observed in many genera (Stebbins, 1974; Igic *et al.*, 2008). In this thesis, I address a series of related questions concerning the common evolutionary transition from SI to SC. In particular, despite years of theoretical as well as empirical work, several unresolved issues remain to be answered regarding the causes and consequences of the maintenance of SI versus SC in angiosperms. To address some of these questions, I use the species *Linaria cavanillesii* as a model system. When starting working on the thesis, *L. cavanillesii* was described as a SC species (Carrió *et al.*, 2013). Hand-pollination in the field as well as preliminary genetic analyses, however, revealed a more complex reproductive biology.

A plant's mating system may be broadly defined as the proportion of selfed vs. outcrossed seeds it produces. The presence of male and female parts on the same flower is widespread in plants, with nearly 90% of angiosperms species being hermaphrodite (Charlesworth 2002) (Figure 1; 2). Although phenological adaptations can promote outcrossing such as temporal (Bertin and Newman 1993; e.g. Kalisz *et al.*, 2012) or spatial separation of male and female function (Webb and Lloyd, 1986; e.g. Takebayashi *et al.*, 2006), selfing can also be prevented by post-pollination physiological mechanisms, which prevent self-fertilization when self-pollen arrive on a stigma (Franklin-Tong, 2008; Shimizu and Tsuchimatsu, 2015). These self-incompatibility (SI) systems are present in approximately 40% of hermaphrodite flowering plant species (Igic *et al.*, 2008, Figure 2), are thought to be ancestral in angiosperms (Allen and Hiscock, 2008), and may have been a key factor facilitating the rapid diversification of flowering plants (Whitehouse, 1950; Barrett, 1998).

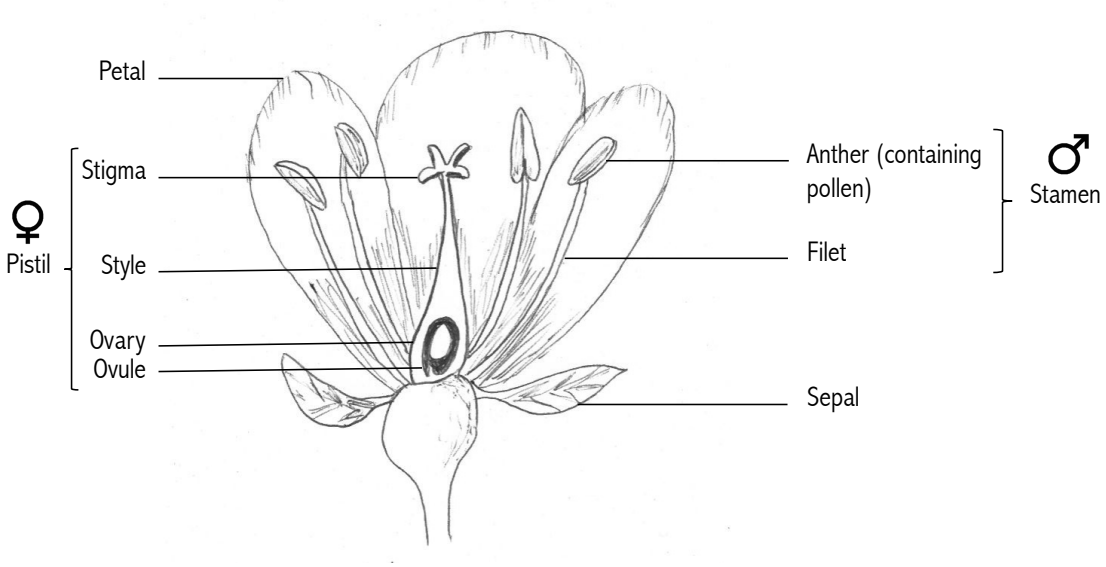


**Figure 1.** One example of a hermaphrodite flower morphology (drawing by the author).

The contrast between SC and SI is often conflated with that between selfing and outcrossing. By definition, SI should cause all a plant's progeny to be outcrossed. However, SC does not mean that all a plant's progeny will result from selfing, and, in extreme cases, SC plants might outcross all their seeds. Of course, many SC plants present some degree of self-fertilization, but it is important to bear in mind that a transition from SI to SC is different from a transition towards complete selfing. A mixed proportion of seeds resulting from selfing and outcrossing is typically referred as "mixed mating" (often characterized as a selfing rate between 0.2 and 0.8; Schemske and Lande 1985), and it has been shown that species presenting a mixed-mating system are common in wild SC populations (42% of 345 SC species tested; Goodwillie et al. 2005) (Figure 2). However, it is not always clear how and why mixed mating is maintained in natural populations (Goodwillie *et al.*, 2005). Similarly, despite the fact that the transition from SI to SC is common in plants (Stebbins 1974; Igic et al. 2008), forces constraining and maintaining mating-system polymorphisms have been difficult to understand. In this context, species presenting among-population polymorphism in SI, or an on-going transition towards SC, are particularly interesting as study systems.

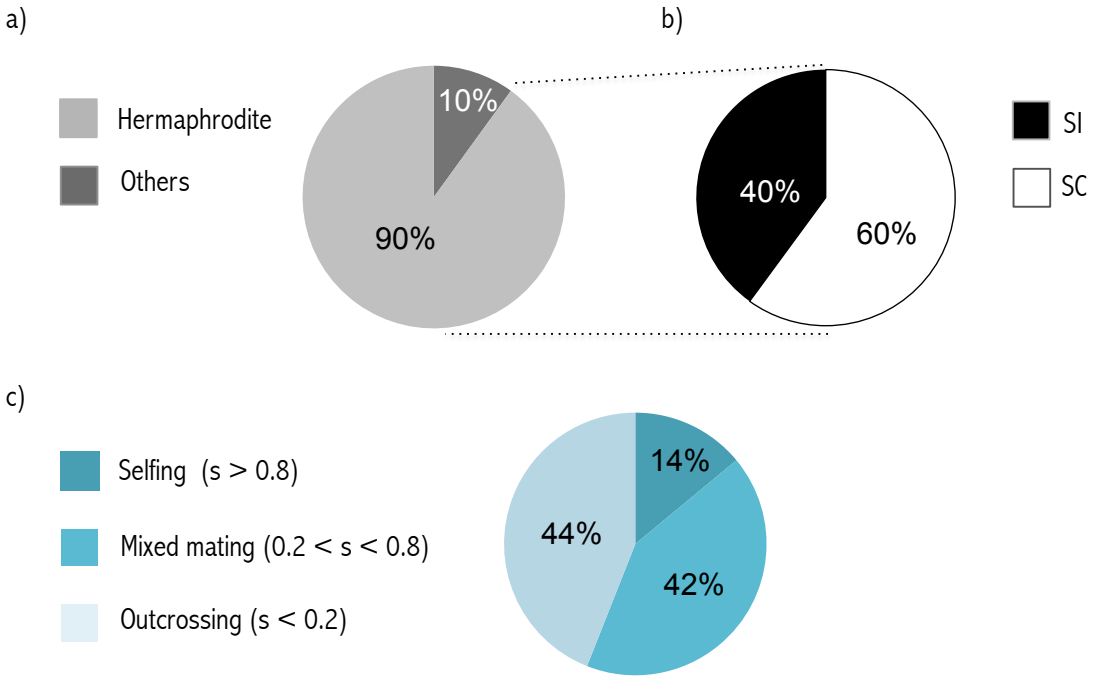


**Figure 2.** The proportion of angiosperms that are (a) hermaphrodite vs. other sexual systems (including mainly dioecious and monoecious species and a minority of other sexual systems); (b) self-incompatible (SI) vs. self-compatible (SC) hermaphrodite species; and (c) mating systems based on selfing rates (s). All percentages are approximations and based on several studies (Barrett, 2002; Charlesworth, 2002; Goodwillie et al., 2005; Igic and Kohn, 2006; Igic et al., 2008).

The first step of my thesis research was to characterize in detail the biology of the perennial herb *Linaria cavanillesii* (Figure 3), starting with the distribution of SI and SC among its populations: which populations are SI, which are SC, and which populations comprise both classes of individual? In a second step, it was important to characterize the patterns of mating within the study populations, using molecular markers to determine the selfing and outcrossing rates in different populations. We found some among-population polymorphisms in mating systems, and indications that the transition from SI to SC was recent. These aspects of my work are presented in **Chapter I**.

One aspect that struck us in the field was the morphological similarity of flowers and inflorescences in the SC vs. SI populations. In theory, when a species or population has shifted to SC and increased selfing rates, we expect to see changes in its floral biology (Ornduff, 1969; Sicard and Lenhard, 2011). Indeed, because selfing species no longer need the presence of pollinators to reproduce, individuals should reallocate resources away from the production of large amount of pollen and structures that attract and

reward pollinators (Charlesworth and Charlesworth 1981). This typically leads to selfing flowers that are smaller, with less pronounced colors, and a reduced production of nectar and pollen (Goodwillie *et al.*, 2010; Sicard and Lenhard, 2011; but see Vos *et al.*, 2014). At first sight, this does not seem to have happened in the SC population of *L. cavanillesii*. Species that have shifted to SC without showing a selfing syndrome may have lost SI recently (e.g. Carleial et al. 2016), or outcrossing may be maintained by selection (e.g. Dart et al. 2012). **Chapter I** thus also includes an analysis of data to determine the extent to which the SC population of *L. cavanillesii* may in fact have taken first steps towards a 'selfing-syndrome'. Contrary to expectations, and in agreement with first observations, I did not find any significant morphological changes in the SC population of *L. cavanillesii*.


**Figure 3.** Illustrations of *Linaria cavanillesii* in Spain: (a) typical natural habitat; (b) a pollinator visiting a flower; (c) bagging treatment used to prevent access to pollinators; (d) flower and branch morphology (photographs by the author).

Interestingly, mixed-mating populations have been shown to harbor similar levels of inbreeding depression compared to their outcrossed counterparts (Winn *et al.*, 2011), which could explain why they do not evolve towards increased selfing rates. Because we observe the maintenance of substantial outcrossing in the SC population of *L. cavanillesii*, as well as an absence of morphological changes towards increased selfing, I investigate in **Chapter II** the levels of inbreeding depression in SI vs. SC populations of *L. cavanillesii*; to understand how much inbreeding depression and purging might explain the maintenance of different mating systems among populations of the same species. In particular, I test the hypothesis that outcrossing might be maintained in the SC population by selection to avoid the expression of inbreeding depression, as seems to explain the maintenance of outcrossing (mixed-mating) in other species (Winn *et al.*, 2011). In fact, although inbreeding depression was high in the SI population sampled, I found an absence of inbreeding depression in the SC population, suggesting that purging has occurred in this mixed-mating population, as it does in highly selfing populations (Husband and Schemske, 1996; Benesh *et al.*, 2014; Noël *et al.*, 2016).

In addition to its effects on inbreeding depression, a transition to increased selfing might also affect patterns of heterosis (the increased fitness of offspring resulting from between-population crosses compared to crosses within populations). Indeed, populations with no inbreeding depression due to purging might still maintain substantial genetic load, because of the fixation of mildly deleterious recessive mutations (for instance, during a population bottleneck associated with the mating-system transition; Kirkpatrick and Jarne 2000), or simply because of a reduced effective population size (Roze and Rousset, 2004). Additionally, populations that have recently shifted to SC usually show stronger population structures because of isolation and reduced size (Hamrick and Godt, 1996; Duminil *et al.*, 2009), which can all increase heterosis between population crosses. In **Chapter II**, I also evaluate the genetic load of one SI and one SC population by comparing heterosis among populations with different mating systems. I found evidence for heterosis in crosses between geographically relatively distant populations, but for SC as well as the SI populations, suggesting that, in addition to the mating system, population size or isolation are crucial parameters too.

The consequences and timing of a transition towards SC have been investigated in a handful of pairs of plant species (e.g. Tang et al. 2007; Foxe et al. 2009; Guo et al. 2009). Additionally, a few studies compared species presenting among-population variation, and these have been particularly useful to understand the dynamics of the breakdown of SI (Foxe *et al.*, 2010; Ness *et al.*, 2010; Busch *et al.*, 2011; Dart *et al.*, 2012). In particular, some species where SI systems have broken down recently show evidence of evolution towards a selfing syndrome, whereas others do not (e.g. Busch 2005; Carleial et al. 2016).

The completion of the two first chapters of this thesis raised some interesting questions about the dynamics of SI breakdown consequences. For instance, the absence of a selfing syndrome in *L. cavanillesii* suggests a recent divergence of the SC population. However, the absence of inbreeding depression in this population remains puzzling, suggesting that purging could occur quickly. In **Chapter III**, in collaboration with two colleagues, I attempt to estimate the age of the breakdown of SI in *L. cavanillesii*. We estimated the divergence time of populations using approximate Bayesian computation methods. Our analysis is consistent with the idea that SC population was recently derived from nearby SI populations.

Despite the abundance of examples where a transition from SI to SC has occurred (Igic *et al.*, 2008), a major gap in our understanding concerns the spread of SC after it has evolved and been fixed in one population. If we recall that plants are sessile, and often grow close to their relatives, SI is a good mechanism to prevent deleterious effect of inbreeding and maintain some levels of genetic diversity. However, once SI has broken down and inbreeding depression has been purged, we end up with SC populations that can benefit from an increase in gene transmissions as well as reproductive assurance. Given these advantages, it remains puzzling that SC does not quickly spread trough the entire species range after it first invades a population: why is SI maintained in the face of potential gene flow from SC populations? Many factors could influence the spread of SC, such as a lower fitness of SC plants due to pollen discounting (reduced outcrossing opportunities due to the use of pollen for self-fertilization; Nagylaki, 1976; Harder and Wilson, 1998; Porcher and Lande, 2005), a lower genetic diversity that can reduce the potential to adapt to new environment (Morran *et al.*, 2009), or the maintenance of some degree of inbreeding depression under mixed mating (Winn *et al.*, 2011). To gain insight into the dynamics of the maintenance of SI vs. SC, I established a multi-generation experiment in which I assessed the likely outcome in the case of invasion of one mating phenotype (SC vs. SI) into an established population containing individuals with the other: the results of this experiment are examined in **Chapter IV**. Additionally, since pollinators have a major influence on mating-system shifts (Eckert et al. 2010; Bodbyl Roels and Kelly 2011; Brys and Jacquemyn 2012; Thomann et al. 2013), I also investigate how this dynamic might be influence by the density of pollinators. Surprisingly, I found that SC plants in mixed arrays had a large fitness advantage over SI plants, both in terms of male and female components of fitness, which resulted in the complete fixation of SC after only three generations of mating.

Finding a significantly higher siring success of SC plants in the experimental mixed arrays runs counter to expectations. Indeed, pollen from selfers and outcrossers are expected to be influenced differently by selection, with stronger selection on pollen competitive ability in outcrossers (Mazer *et al.*, 2010). This is in part because outcrossing flowers are usually larger and more attractive to pollinators (Goodwillie and Ness, 2005), leading to the receipt of many pollen grains that have to compete to grow down longer styles, thus

selecting for high pollen performance (Mazer *et al.*, 2010). In contrast, selection for high performance is expected to be reduced in selfers because antagonistic selection is reduced in mating events where male and female components are not in conflict (Kerwin and Smith-Huerta, 2000). Under this scenario, one hypothesis states that crosses with plant of different mating systems should result in higher siring success for outbred parents (Brandvain and Haig, 2005). Thus, to understand how much SC vs. SI plants might vary in their ability to sire offspring, I set up an experiment in **Chapter V** with the help of a master student. We applied a different mix of pollen on stigmas, and paternity was estimated for the offspring. In contrast with theoritical expectations, we found that SC pollen had better competitive ability, by siring more offspring, than SI pollen. We suggest that this pollen competitive ability difference may have helped the spread of diffusion of SC in our mixed arrays.

In summary, the chapters of my thesis that follow below thus report a series of investigations that begin with the observation of the loss of SI in part of the range of an otherwise outcrossing species (**Chapter I**). I ask what might allow the maintenance of SI and SC in populations in which these contrasting systems are found (**Chapter II**) and attempt to determine the age of the transition from SI to SC (**Chapter III**). Finally, I follow the dynamics of an experimentally established within-population polymorphism in SI vs. SC to understand how the fixation of SC in one population might, or might not, spread through the rest of a species range (**Chapter IV**). I follow up on this experiment by assessing the extent to which differences in the competitive ability of pollen grains dispersed by SC vs. SI plants might help to explain the rapid spread of SC observed in the experimental populations (**Chapter V**). In each chapter, I present a more detailed background to the hypotheses tested to that given in this short introduction here, and discuss their wider implications.

# - CHAPTER I -

## Maintenance of mixed mating after the loss of self-incompatibility in a long-lived perennial herb

M. Voillemot and J. R. Pannell

*(Published in Annals of Botany, 2016, 119(1), pp. 177-190)*

# ABSTRACT

Many hermaphroditic plants avoid self-fertilization by means of a molecular self-incompatibility (SI) system, a complex trait that is difficult to evolve but relatively easy to lose. Loss of SI is a pre-requisite for an evolutionary transition from obligate outcrossing to self-fertilization, which may bring about rapid changes in the genetic diversity and structure of populations. Loss of SI is also often followed by the evolution of a 'selfing syndrome', with plants having small flowers, little nectar and few pollen grains per ovule. Here, we document the loss of SI in the long-lived Spanish toadflax *Linaria cavanillesii* that has led to mixed mating rather than a transition to a high rate of selfing and in which an outcrossing syndrome has been maintained. We performed crosses within and among six populations of *L. cavanillesii* in the glasshouse, measured floral traits in a common-garden experiment, performed a pollen-limitation experiment in the field, and conducted population genetic analyses using microsatellites markers. Controlled crosses revealed variation in SI from fully SI, through intermediate SI, to fully self-compatible (SC). Flowers of SC individuals showed no evidence of a selfing syndrome. Although the SC population of *L. cavanillesii* had lower within-population genetic diversity than SI populations, as expected, population differentiation among all populations was extreme and represents an $F_{ST}$ outlier in the distribution for both selfing and outcrossing species of flowering plants. Together, our results suggest that the transition to SC in *L. cavanillesii* has probably been very recent, and may have been aided by selection during or following a colonization bottleneck rather than in the absence of pollinators. We find little indication that the transition to SC has been driven by selection for reproductive assurance under conditions currently prevailing in natural populations.

**Keywords:** fruit set, genetic diversity, *Linaria cavanillesii*, outcrossing, pollen limitation, reproductive assurance, selfing syndrome

# INTRODUCTION

Most flowering plants are hermaphroditic, with both male and female functions in the same flowers (Darwin 1877; Barrett 2002; Charlesworth 2002). Although hermaphroditism may allow self-fertilization, numerous strategies have evolved to promote outcrossing (Darwin 1877; Barrett 2003; Karron et al. 2012). These strategies include both morphological and phenological adaptations involving the spatial (Webb and Lloyd 1986) and temporal separation (Bertin and Newman 1993) of the two sexual functions, as well as molecular self-incompatibility (SI) mechanisms that prevent fertilization following self-pollination (Franklin-Tong 2008; Shimizu and Tsuchimatsu 2015). Yet the loss of outcrossing mechanisms such as SI represents perhaps the most frequent shift to have taken place in angiosperm evolution (Stebbins 1974; Igic et al. 2008). Seeking reasons for these shifts, and understanding their consequences, have been pre-eminent goals in our quest to understanding plant reproductive evolution (Barrett 2002).

Two main reasons have been hypothesized for the breakdown of outcrossing mechanisms in plants. First, because selfing immediately allows a plant to transmit two copies of its genome to its seed progeny rather than just one, there should be automatic selection of mutations that increase the frequency of selfed progeny (Fisher 1941) – as long as the quality of offspring are not overly compromised by inbreeding depression (Charlesworth and Charlesworth 1987) and selfing does not compromise outcross siring success (i.e., there is no pollen discounting; Harder and Wilson 1998). The maintenance of outcrossing in the face of automatic selection for selfing likely attests to the high levels of inbreeding depression that characterize outcrossing species (Lande and Schemske 1985; Husband and Schemske 1996; Goodwillie et al. 2005; Winn et al. 2011).

Second, selfing may be selected under conditions where outcrossing is difficult. Being able to self-fertilize allows a plant to reproduce in the absence of mates or pollinators (Jain 1976; Lloyd and Schoen 1992).

Reproductive assurance provides the most plausible reason for the breakdown of outcrossing mechanisms, and there is growing empirical support for it (Herlihy and Eckert 2002; Elle and Carney 2003; Kalisz and Vogler 2003; Moeller and Geber 2005; Eckert et al. 2006; Busch and Delph 2012). For example, Kalisz and Vogler (2004) found an increase in the rate of selfing and fruit production in the herb *Collinsia verna* in conditions of low pollinator availability. The enrichment of self-compatibility (SC) on islands colonized by long-distance dispersal provides indirect support for the loss of outcrossing mechanisms in the absence of mates (Baker 1955, 1967; McMullen 1987; Webb and Kelly 1993), as does the observation of increased selfing at the margins of a species' range (Busch 2005; Michalski and Durka 2007; Mimura and Aitken 2007), and the greater range sizes of selfing species than their outcrossing relatives (Grossenbacher et al. 2015; Pannell et al. 2015; reviewed in Pannell 2015).

Whatever its initial cause, once a transition to increased self-fertilization has occurred, we expect rapid changes in the distribution of genetic diversity (Hamrick and Godt 1996; Wright et al. 2008, 2013) and floral traits (Ornduff 1969). Because a transition to selfing increases homozygosity and reduces effective recombination (Nordborg and Donnelly 1997), we expect and observe a loss of diversity within populations (Hamrick and Godt 1996; Charlesworth and Pannell 2001), an increase in genetic differentiation among populations (Glémin et al. 2006; Duminil et al. 2009), and changes in genome organization and sequence, such as a reduction in genome size or an increased linkage disequilibrium (Wright et al. 2008; reviewed in Shimizu and Tsuchimatsu 2015). Transitions to selfing can also reduce the capacity of a population to respond to selection in a changing environment (Morran et al. 2009), potentially providing an explanation for the observed increase in extinction probability of plant lineages that have lost their SI system (Stebbins 1974; Wright et al. 2007; Goldberg et al. 2010; Wright and Barrett 2010).

Species that have evolved a selfing strategy typically also show changes in the size, morphology and phenology of their flowers (Ornduff 1969; Fenster and Armbruster 2004; Goodwillie et al. 2010; Sicard

and Lenhard 2011). Plants that no longer rely on pollinators for their reproduction should reduce allocation to pollinator attraction and reward through decreases in flower size, floral scent and nectar production, as well as in the ratio of pollen grains to ovules (Cruden 1977; Charlesworth and Charlesworth 1981). This 'selfing syndrome' has been observed frequently in populations with increased selfing rates (Goodwillie et al. 2010), and can evidently evolve rapidly (Bodbyl Roels and Kelly 2011). Bodbyl Roels and Kelly (2011) prevented pollinators from accessing flowers in experimental populations of the SC plant *Mimulus guttatus* and observed reduced anthers-stigma separation in only five generations, and several studies have shown similarly rapid changes in floral size in response to artificial selection (Worley and Barrett 2000; Lendvai and Levin 2003; Delph et al. 2004). Given the rapidity of such responses, we should perhaps not be surprised that a selfing syndrome was able to evolve following the loss of SI in the selfing species *Capsella rubella* over several thousand generations (Foxe et al. 2009; Slotte et al. 2012).

Although apparently common, the evolution of a selfing syndrome following a loss of an outcrossing mechanism is not inevitable (e.g. Busch 2005; Zhang et al. 2005; Fenster and Martén-Rodríguez 2007; Dart et al. 2012). Indeed, Fenster and Martén-Rodríguez (2007) reported that many species with relatively high selfing rates nevertheless maintain many of the hallmarks of outcrossing, including showy flowers, high pollen/ovule ratios, and substantial nectar production. Most of these species appear to have specialized pollinators, and almost all show delayed selfing, with self-fertilization occurring when opportunities for outcrossing have been exhausted (Schoen and Lloyd 1992). As Fenster and Martén-Rodríguez (2007) point out, such a syndrome of SC, intermediate selfing rates, and traits otherwise associated with outcrossing probably represents a response to selection for reproductive assurance in populations that usually enjoy high pollinator visitation. However, it is less obvious why SC species that fail to delay their self-fertilization should maintain attractive flowers, particularly in those lineages that were previously SI (though see below).

Traits promoting outcrossing appear to have been maintained in a number of species despite loss of SI. For example, outcrossing and the attraction and reward of pollinators appear to have been maintained in some populations of the plant *Leavenworthia alabamica* following the loss of SI, despite the absence of a delayed mode of self-fertilization, whereas other populations of the same species have evolved a selfing syndrome (Lloyd 1965; Busch 2005). Similarly, North American populations of *Arabidopsis lyrata* that have lost SI continue to display flowers with no reduction in size and no evidence of traits that would promote only delayed selfing (Mable et al. 2005; Hoebe 2009), as have a number of populations of the North American plant *Camissoniopsis cheiranthifolia* (Raven 1969; Dart et al. 2012). The loss of SI in these species has neither led to the evolution of selfing and a selfing syndrome, nor to the evolution of mechanisms that would delay selfing under continued investment in outcrossing.

The examples just cited represent revealing case studies of within-species variation in annual or short-lived perennial plants in which SI has broken down and populations have come to differ in their mating strategies. Here, we document the breakdown of SI in the long-lived perennial toadflax *Linaria cavanillesii* in which there also appears to have been no accompanying transition towards a selfing syndrome. *L. cavanillesii*, a plant endemic to south-eastern Spain, was recently described as SC on the basis of crossing experiments performed on one of its populations (Carrió et al. 2013). We were surprised by this finding, as individuals produce large, showy flowers with copious nectar. Our investigations of other populations of the species reveal that the species is in fact largely strongly SI, likely possessing the same gametophytic SI system that is widespread in the genus *Linaria* and its family Plantaginaceae (Pandey 1960; Franklin-Tong 2008). We hypothesized that SC in *L. cavanillesii* represents a potentially recent loss of this SI mechanism in part of the species' range.

Our main aim in this paper is to characterize variation in the mating system and floral biology of *L. cavanillesii* across its range in south-eastern Spain, and to investigate the morphological and genetic consequences of a transition from SI to SC. We were particularly interested in understanding why an

outcrossing syndrome appears to be maintained in populations that have lost SI. We used hand-pollination experiments in the glasshouse to ascertain the extent to which individuals of *L. cavanillesii* are capable of self-fertilization, and whether self-fertilization could occur autonomously in the absence of pollinators. We assessed the extent to which *L. cavanillesii* shows patterns of genetic diversity within and between populations that are consistent with expectations of a transition from outcrossing to self-fertilization, and whether they have begun to evolve any characteristics of a selfing syndrome. Finally, we asked whether reproductive assurance could have resulted in the transition to SC in this mostly outcrossing species. Comparison of our results with those of other species reveals that *L. cavanillesii* occupies a relatively extreme combination of high genetic differentiation among populations despite high outcrossing rates.

# MATERIALS AND METHODS

*Site and study species*

*Linaria cavanillesii* (Plantaginaceae) is a perennial herb endemic to southeastern Spain with a protected conservation status, mainly due to the high fragmentation of populations, as well as the low number of individuals in each populations (Sánchez-Gómez et al. 2003; Cabezudo et al. 2005). It grows along north-northwest-oriented cliffs at sites ranging from 300 to 1400 m in altitude (Sutton 1988; Laguna et al. 1998). In spring, individuals grow multiple herbaceous shoots from a sub-woody perennial base, with inflorescences produced towards the end of the branches. Flowers are yellow, with a floral nectar spur that is typical of the genus, and are pollinated by a variety of bees and bumblebees. Flowering occurs between May and June. Around 30 days after fertilization, capsules open and seeds are dispersed passively, probably aided by wind. Using herbarium records to locate populations of *L. cavanillesii,* we were able to find 11 extant populations, all in the Alicante-Murcia region of southeastern Spain. Our study was carried out in six of these populations that were sufficiently accessible for study (individuals grow on rock faces and are often out of reach; Figure 1; Table S1).

*Hand-pollination crosses*

To characterize the distribution of self-incompatibility (SI) and self-compatibility (SC) within and among populations, we carried out hand-pollination crosses on plants grown in glasshouses at the University of Lausanne, Switzerland, from seeds collected in the field. Experimental crosses were performed mostly between November 2012 and March 2013. Five treatments were applied to flowers: (1) autonomous pollination (inflorescences were bagged with small fine-meshed nylon bags at the bud stage few days before flowers opened, and flowers and eventually fruits were allowed to develop on their own); (2) self-pollination (inflorescences were bagged at the bud stage, anthers were removed prior to anthesis with fine forceps, and self pollen was applied to stigmas); (3) outcrossing within populations (inflorescences were bagged at the bud stage, anthers were removed prior to anthesis, and outcross pollen from another plant in the same population was applied to stigmas); (4) outcrossing between populations (inflorescences were bagged at bud stage, anthers were removed prior to anthesis, and outcross pollen from a donor plant in another population was applied to stigmas); and (5) a control cross (inflorescences were bagged at the bud stage, anthers were removed prior to anthesis with fine forceps, and no further manipulation was performed). Two days after flowers opened, pollen was applied by gently brushing an anther against a stigma until it was covered by pollen. Additionally, we also tested delayed selfing by applying pollen to flowers five days after opening. We assessed fruit development after 14 days. We applied rubber glue to the tips of developing fruit capsules approximately 18 days after pollination (when fruits were fully formed) to prevent the loss of seeds upon fruit dehiscence. We collected fruits approximately ten days after gluing, at which point they were fully mature, and we counted and weighed seeds. Each treatment was performed once on different flowers for each individual, with a total of twenty different plants from each population.

*Fruit set and pollen limitation experiment*

To assess whether individuals in the SC population enjoy the benefit of reproductive assurance, we estimated the proportion of flowers setting fruits (fruit set) in the field. In *L. cavanillesii*, fruits that contain viable seeds are much larger than those that have not been pollinated, and can thus be easily distinguished. Because capsules remain open on the plant after seed dispersal, without dropping off, fruit set can be estimated even after seed dispersal. We took measurements in each population on as many plants as were available in both 2013 and 2015 (sample sizes are given in the Results).

We also performed a pollen limitation experiment in the field in May 2015. In each population, we randomly selected plants and applied the four following treatments on marked flowers: (1) control (inflorescences left unmanipulated with no bagging); (2) control bag (inflorescences bagged at bud stage, and flowers left unmanipulated); (3) supplementary outcross pollen (anthers removed prior to anthesis, and, when mature, outcross pollen applied to stigmas from at least two donor plants from the same population); and (4) emasculation (no bagging, anthers removed prior to anthesis, and the flowers left unmanipulated). Control and supplementary outcross treatments are used to compare pollination success under natural conditions or under conditions of pollen supplementation; and emasculation treatments to assess outcross pollen limitation. Control bag treatments were used to identify plants that have an ability to self-fertilize autonomously without the aide of pollinators. Finally, we assessed fruit development after 14 days.

*Phenotypic comparison of plants among populations*

To assess the degree to which the loss of SI might have precipitated the evolution of a selfing syndrome, we compared flowers from the single known fully SC population with those from three other populations comprising SI individuals. For each population, we conducted measurements on 30 individuals grown from seeds, each produced by a different individual sampled in the field. Seeds were first cleaned with

bleach and germinated in Petri dishes placed in phytotron (conditions: 13 h light/11 h dark, 20:15°C day:night, 80 % humidity), before transplantation into pots. Plants were grown in a randomized block design on glasshouse benches, with five plants from each population placed in each of six blocks. Plant positions were randomized once a week.

We measured the following traits for each individual: number of days to germination, total number of germinated seeds, plant size after 25 days and 16 weeks of growth, number of days to flowering, total number of flowers produced over three months of flowering, flower longevity, flower (length from the top of the corolla to the tip of the spur) and spur size (length from the beginning to the tip of the spur), nectar quality (measured with two refractometers to cover a range of nectar concentration of 0-50 Brix and 45-80 Brix; Bellingham & Stanley Ltd, Tunbridge Wells, UK) and pollen/ovule ratio. In order to account for flower variability within plants, every phenotypic measure of flowers was taken and averaged using at least three flowers per plant, and flower longevity was estimated for at least two flowers per plant. Due to some plant mortality, every measure was taken on an average of 19 plants per population (min: 16; max: 23).

We estimated the number of pollen grains using a particle counter (Elzone II 5390 Micromeritics®). For each sample, one upper and one lower anther of a non-opened flower were fixed in formaldehyde-acetic acid alcohol solution (FAA; 5 parts glacial acetic acid: 5 parts 38% formaldehyde: 90 parts 70% ethanol). Before analysis, samples were sonicated (Branson 52, Emerson industrial automation) for two minutes to release pollen from anthers, and then transferred into the analysis beaker of the particle counter which contained 100 mL of dd$H_2O$ with 2% NaCl. Each sample was analyzed four times for 30 s, and an average of the four replicates was used to estimate the number of pollen grains per anther. We estimated pollen production for each plant as an average of measures taken for three flowers per plant. For each population, the diameter range of pollen grains was estimated using photos taken with a microscope equipped with a digital camera (sample sizes: 67 to 93 pollen grains per population; Olympus DP-10 digital camera,

Hamburg, Germany). We estimated the number of ovules for the same flowers used for pollen counting, based on floral dissections using a razor blade and counted under a binocular microscope (Leica MZ 125, Leica Microsystems ®).

*Genetic diversity and selfing rates estimation*

To estimate genetic diversity and differentiation among populations, as well as their respective selfing rates, we assayed variation at 16 microsatellite loci (Note S1). We collected leaf material from the six natural populations (sample sizes: 29 – 41 per population), and extracted DNA with the DNeasy 96 Plant kit (Qiagen). Polymorphic primers (Note S1) were amplified by PCR (Biometra thermocycler; Göttingen, Germany) using the following reagents: 1xPCR mix: 2 ng/µL template DNA, 10x PCR Buffer, 25 mM $MgCl_2$, 5x Q-solution, 2.5 mM dNTP, 0.2 µM of each primer and 0.5 U/µL of Taq DNA polymerase (HotStarTaq®, Qiagen). The thermocycling conditions were 15 min at 95° followed by 32 cycles of 30 sec at the annealing temperature (Note S1; Table S2), 30 sec at 72 °C and 30 sec at 95 °C, followed by one cycle of 1 min at the annealing temperature (Note S1; Table S2) and a final extension of 30 min at 72 °C. PCR products were then sequenced in an ABI3100 (Applied Biosystems). We used the program Genemapper® to analyze microsatellites data, and the program Fstat (Goudet 1995) to calculate and compare genetic diversity, allelic richness, population structure ($F_{ST}$) and inbreeding coefficient ($F_{IS}$) among populations. We estimated selfing rates using the software RMES (David et al. 2007), which infers selfing rates from the distribution of multilocus heterozygosity taking into account the possible presence of null alleles.

*Statistical analyses*

We used linear mixed models to investigate phenotypic differences among populations, using the "lme4" package in R (Bates et al. 2014), with 'population' as a fixed effect and 'block' as a random factor in order to account for spatial variation. For the assessment of phenotypic differences, we included the date

of measurement as an additional random factor. Significance of variables was analysed using a stepwise deletion procedure, and multiple comparisons of means were performed using the "lmerTest" package in R (Kuznetsova et al. 2014). For binomial data (i.e., proportion of germination or fruit set), a generalized linear model (glm) was used with quasibinomial errors. If significant, differences within treatments were then tested by means of post-hoc tests, using the 'glht' function of the multcomp package in R. Measures of allelic richness and genetic diversity were compared among populations using analysis of variance, with differences among populations tested using post-hoc Tukey HSD tests. All analysis reported were conducted in R (version 3.1.2, R Core Team 2015).

# RESULTS

*Fruit set and seed production following hand-pollination in the greenhouse*

Experimental hand-pollination revealed variation in selfing ability among the populations. In total, 466 crosses were performed. The outcross treatment (within and among populations) produced fruit consistently at 91% (successful fruit formation/number of crosses: 205/226, Figure S1). Self-pollination resulted in total fruit production in one population (COV) and in moderate fruit production (<35%) in three populations (BUI, RUB, ZAR; Figure S1). Additionally, the rate of autonomous selfing was high in the COV population (>80%) and moderate in the BUI, RUB and ZAR populations (<15%; Figure S1). No plants showed any evidence of delayed selfing.

Seed production varied substantially among capsules (95 ± 40 seeds per fruit; minimum: 4, maximum: 180; averages taken across populations), and among individuals and populations (Figure 2). In summary, two populations (DEN, BER) exhibited strong SI, with no or negligible seed production upon selfing by any individuals tested; three populations (BUI, ZAR, RUB) showed weaker SI, with most plants being strongly SI and a few showing 'leaky' SI but still producing fewer seeds after self vs. outcross treatments;

and only one population (COV) showed full SC, with all individuals tested producing approximately the same number of seeds in the self vs. outcross treatments (Figure 2).

*Variation in fruit set under open-pollination in the field*

Observations in the field revealed variation in fruit set among populations, ranging from 0.48 (BER) to 0.90 (ZAR; Figure 3). Averaged over the two years of the study, SI populations and mixed populations showed a lower fruit set compared to the SC population (SI: 0.62 ± 0.06; mixed: 0.77 ± 0.03; SC: 0.85 ± 0.03, Figure 3), with significant differences between the SC population and three other populations (glm: $F_{5, 212} = 18.46$, $p < 0.001$; post-hoc test: COV-BER: $p < 0.001$; COV-BUI: $p < 0.001$; COV-RUB $p < 0.001$).

In contrast with other populations, certain treatments were problematic for the BUI population, with the supplementary pollination treatment yielding substantially less than 100% fruit set. Control flowers did not develop into fruits, preventing any valuable comparison among treatments. We thus excluded this population from further analyses. In the other populations, the supplementation of pollen led to consistently high fruit set (average: 91% ± 0.10), As expected, only the SC population showed consistent fruit set in the bagged treatment with no access to pollinators (COV, n = 18/19). Fruit set in the emasculation treatment was somewhat lower than the pollen supplementation treatment for the SC population and one mixed populations (COV: -3%, ZAR: -9%); but was substantially lower for the two other SI populations and one mixed population (BER: - 46%, DEN: -80%, RUB: - 41%). These results point to strong pollen limitation in these populations (BER, DEN, RUB; Figure 4), and to an absence of pollen limitation in the two other populations (COV, ZAR, Figure 4). This trend was similar when comparing fruit set in the control treatment to that in the pollen supplementation treatment (COV: - 7%, ZAR: -5%, DEN: -40%, BER: -35%, RUB: -13%; Figure 4).

*Phenotypic variation among populations*

Populations varied for a number of plant traits, with a significant effect in eight of the eleven traits measured (non-significant traits were flower longevity, pollen/ovule ratio, and size after 25 days; Table 1; Figures S2-S5). The mean number of days to germination as well as nectar quality were significantly different between one SI population (ZAR) and all the other populations (Table 1; Figure S2). However, no significant differences were consistently found among the other populations (Figures S2-S5). We found little evidence of trait variation consistent with evolution towards a selfing syndrome in the SC population, except that plants in the SC population showed a somewhat shorter time to flowering (Table 1; Figure S4).

*Genetic diversity, levels of inbreeding and population genetic structure*

We found significant differences in measures of genetic diversity between the SC and SI populations (Table 2). In particular, while one SI (ZAR) population showed a negative $F_{IS}$ (-0.047), all other populations showed positive $F_{IS}$ (Table 2). After correction for multiple testing (corrected p-value = 0.0005), only one leaky SI (RUB) and the SC (COV) population showed a significantly positive $F_{IS}$ (Table 2).

Allelic richness was lower for the SC population compared to all the other populations, and this difference was significant for one SI population (DEN; Anova: $F_{5,90} = 2.34$, $p = 0.05$, TukeyHSD: $p < 0.048$; Table 2). Genetic diversity showed the same pattern, with a lower diversity for the SC population, which differed significantly from two other populations (BER and ZAR; Anova: $F_{5,91} = 2.73$, $p = 0.02$, TukeyHSD: $p < 0.05$; Table 2).

Population genetic differentiation was generally high, being strongest among the three northern-most and the three southern-most populations ($F_{ST} > 0.70$), and lowest among the three southern-most populations

($F_{ST}$ < 0.35, Table 3). Differentiation between the SC population and the two SI populations in the north was also strong ($F_{ST}$ > 0.56), and somewhat weaker between the two SI populations ($F_{ST}$ = 0.44, Table 3).

*Variation in selfing rates*

The inferred selfing rates varied among the six populations, with the five largely SI populations showing predominant outcrossing ($s$ < 0.2), and the SC population showing mixed mating ($s$ = 0.59, falling within the range between 0.2 and 0.8 commonly regarded as mixed mating; Schemske and Lande 1985; Table 2).

# DISCUSSION

Our study documents variation in self-incompatibility and the mating system among populations of the Spanish long-lived perennial herb *Linaria cavanillesii*. This species was hitherto described as self-compatible (SC) (Carrió et al. 2013), but our results indicate that it is in fact largely a self-incompatible (SI) outcrossing species that has likely undergone a shift to SC in the northern extreme of its range. Self-incompatibility in the SI populations was generally strong, but some populations showed evidence for leaky expression of SI. Although plants in the SC population could benefit from reproductive assurance in the absence of pollinators, we found that pollinator activity was generally sufficient to ensure reproduction. Moreover, although the fully SC population showed some of the population-genetic hallmarks of a history of selfing, it has strikingly retained floral traits characteristic of an outcrossing syndrome, and its outcrossing rate remains moderately high. Interestingly, both the SC and SI populations of *L. cavanillesii* represent outliers in the plane relating the realized mating system to patterns of population genetic differentiation, with particularly high $F_{ST}$ values found among populations. Below, we discuss the significance of these findings, emphasizing what they contribute to an understanding of the drivers and consequences of the loss of SI in flowering plants. In particular, we consider what uncommon aspects of the biology of *L. cavanillesii* might be responsible for the unusual patterns it shows in terms of its floral biology and genetic variation.

*Variation in self-compatibility among populations*

Our study documents a new example of within-species variation in SI in a flowering plant. Crosses in the greenhouse revealed that of the six isolated populations sampled, two are strongly SI (with all individuals tested being incapable of selfing), one is fully SC (with all individuals capable of autonomous selfing), and three are variable in their self-compatibility, with SI and a few SC plants occurring. Several cases of such within-species variation have been investigated in some detail, most of which are annuals or short-lived perennial species (e.g. Lloyd 1965; Raven 1969; Busch 2005; Mable et al. 2005; Dart et al. 2012). *L. cavanillesii* is a long-lived perennial plant in which an ability to self-fertilize is likely to be less important for reproductive assurance (Baker 1955; Pannell and Barrett 1998; Aarssen 2000; Larson and Barrett 2000), and in which selection to avoid inbreeding depression caused by selfing should be particularly high (Duminil et al. 2009; and see below).

Not only does *L. cavanillesii* vary among populations in the strength of SI, individuals also vary within some populations. In these populations, a few plants that we isolated from pollinators in a glasshouse occasionally produced fruits by autonomous selfing, albeit with fewer seeds than those that had been outcrossed. The sporadic production of a low proportion of seeds upon self-pollination has been shown in many SI species and has been referred to as 'leaky SI' or 'pseudo-self-incompatibility' (Levin 1996) (e.g. Brennan et al. 2005; Busch 2005; Crawford et al. 2008, 2010; Dart et al. 2012; Zhang et al. 2014). As a transitional state, leaky SI may have played a role in the transition from SI to full SC in some species (Good-Avila and Stephenson 2002), as it may have done in loss of SI in at least one population of *L. cavanillesii.* It is noteworthy that the transition to full SC has occurred in only a small part of the species range, despite the occurrence of leaky SI in other populations. In these populations, the fact that inbreeding depression is high (Chapter II) may have selected against further independent transitions to SC. Strong inbreeding depression is likely to have been a key factor maintaining SI system in the colonizing

species *Senecio squalidus*, where approximately 3% of wild plants investigated show leaky SI (Brennan et al. 2005).

*Variation in the mating system among populations*

Despite the possibility of occasional selfing in populations of *L. cavanillesii* with leaky SI, our estimates of the inbreeding coefficient of their adult plants point either to very limited selfing in the wild, or to strong viability selection against selfed progeny. Either way, it is clear that the occasional ability to self-fertilize in some populations of *L. cavanillesii* has not affected the realized mating system very much. In contrast, high inbreeding coefficients estimated for the fully SC population indicate that it has intermediate realized selfing rates (an inferred selfing rate of $s = 0.59$), i.e., this population appears to have a mixed mating system. The finding of substantial numbers of inbred adults in the SC population suggests that inbreeding depression must be sufficiently low to allow at least some selfed individuals to reach reproductive maturity. Direct estimates of inbreeding depression for this population (Chapter II) are consistent with this inference.

Although mixed mating (commonly defined for $0.2 < s < 0.8$; Schemske and Lande 1985) is quite common in flowering plants (Vogler and Kalisz 2001; 42% out of 345 plant species tested: Goodwillie et al. 2005), our finding of substantial outcrossing in the SC population of *L. cavanillesii* is somewhat surprising. While outcrossing may be maintained in SC species through other outcrossing mechanisms, such as the temporal (Bertin and Newman 1993, e.g. Kalisz et al. 2012) or spatial separation of the sexes within flowers (Webb and Lloyd 1986; e.g. Karron et al. 1997; Takebayashi et al. 2006), the stigmas of *L. cavanillesii* are fully surrounded by, and in contact with, the anthers, and they appear to be receptive as soon as anthers dehisce. We might thus have expected SC individuals to self-fertilize all their ovules autonomously, precluding opportunities for outcrossing. Even if plants are indeed able to self-fertilize autonomously, we found that the flowers of SC individuals of *L. cavanillesii* are as large and rewarding as those of SI individuals, and they continue to be visited by pollinators. The maintenance of substantial

levels of outcrossing despite the loss of SI thus points either to a 'competing' or a 'delayed' mode of selfing in *L. cavanillesii* (see Lloyd and Schoen 1992). In discussing their survey of the literature, Fenster and Martén-Rodríguez (2007) suggested that species in which SC individuals continue to attract and reward pollinators seemed mainly to be capable of delayed selfing, a device that promotes outcrossing while nevertheless assuring reproductive assurance via selfing when pollinators are absent. Specifically, they found that 84% of 38 SC species investigated displayed delayed selfing, with only 16% showing prior or competing selfing. Of these, half showed competing selfing with protandrous flowers, and half showed no temporal separation between male and female functions within flowers. A particularly interesting example is provided by the species *Camissoniopsis cheiranthifolia,* a species with mixed mating in which the selfing rate is associated with differences in floral morphology among populations (Dart et al. 2012). In *C. cheiranthifolia*, as in *L. cavanillesii*, the fact that flowers remain attractive and are visited by pollinators accounts for the maintenance of substantial outcrossing, despite an ability of individuals to self. Similarly, in populations of the annual plant *Collinsia verna*, high pollinator visitation ensures high levels of outcrossing despite the ability to self autonomously (Kalisz and Vogler 2003, 2004).

*Pollen limitation and reproductive assurance*

Results of our pollen supplementation experiment, as well as global fruit set observed at the end of each of two reproductive seasons in the field, revealed variation among populations in the extent to which their seed set was pollinator-limited. Whereas plants in the fully SC population were able to set fruit autonomously in the absence of pollinators, pollinator visitation levels were such that this ability was not necessary for high seed set, as indicated by substantial seed production by emasculated flowers. The same was found in one of the SI populations of *L. cavanillesii*, in which pollinator visitation ensured seed set equivalent to that produced by hand-pollinated flowers. Our results thus provide little support for the importance of selection for reproductive assurance in driving the loss of SI in the northern extreme of the range of *L. cavanillesii*, where the SC population is found. This conclusion contrasts with that reached for other species, in which pollen limitation appears to be reduced in geographically marginal populations that

have evolved selfing (Busch 2005; Michalski and Durka 2007; Mimura and Aitken 2007; but see Herlihy and Eckert 2005; Moeller et al. 2012).

Although pollen limitation and selection for reproductive assurance may be of limited importance in the northern range of *L. cavanillesii*, pollinator visitation was insufficient to ensure full seed set in four of the SI populations investigated, where pollen supplementation substantially increased seed set. Although results of pollen supplementation tests must be interpreted with caution in absolute terms, and perhaps conservatively (Ashman et al. 2004; Knight et al. 2005), it nevertheless seems clear that these SI populations of *L. cavanillesii* were at least more pollen-limited than the SC and one other SI population that showed an absence of pollen limitation. Only in these four SI populations might one thus expect selection potentially to favour mutations conferring SC and the evolution of selfing as reproductive assurance (Lloyd and Schoen 1992). As noted above, however, inbreeding depression might be sufficient to maintain outcrossing despite this potential advantage, as argued for the mixed mating species *Aquilegia canadensis* (Herlihy and Eckert 2002).

It is widely thought that selection for reproductive assurance in the face of pollen limitation is likely to be strongest in short-lived or annual species, in which reproductive failure is particularly consequential (Larson and Barrett 2000; Duminil et al. 2009). Given that *L. cavanillesii* is a long-lived perennial, we should therefore not expect selection for reproductive assurance to be particularly important, even in those populations that were somewhat pollen-limited. Of course, our field observations were carried out over only two reproductive seasons, and pollinator activity might have been higher in other years in the pollen-limited SI populations. Nevertheless, it does seem unlikely that transition to SC was driven by selection for reproductive assurance under conditions we observed in the SC population. Rather, reproductive assurance may have been an important selective factor when that population was first colonized, e.g., through mate limitation (see below).

*Variation in genetic diversity and differentiation among populations*

We found that the SC population was genetically less diverse than the SI populations. This pattern is a typical finding in comparisons between selfing and outcrossing species or populations (Hamrick and Godt 1996; Mable et al. 2005; Glémin et al. 2006), and is consistent with theoretical expectations (Charlesworth 2003). We also found strong differentiation between northern and southern populations, and particularly between the SC population and all the other populations. Again, this is consistent with the observation that selfing populations tend to be more strongly differentiated from one another than outcrossing populations (Hamrick and Godt 1996; Duminil et al. 2009). Low genetic diversity in selfing populations has been generally attributed to their lower inbreeding effective sizes (which, for fully selfing populations, should be 50% the effective size of a fully outcrossing population; Pollak 1987; Nordborg and Donnelly 1997), and thus increased effects of drift locally. It is difficult to estimate the effective size of the populations we sampled, but the current census size of the selfing population is probably substantially greater than the size of most of the outcrossing populations we sampled (M. Voillemot and J.R. Pannell, personal observation). Given that the selfing rate in the SC population is intermediate, we doubt that the short-term effects of increased inbreeding are sufficiently severe to account for the lower diversity found.

The higher levels of genetic differentiation generally observed among selfing populations (Hamrick and Godt 1996; Duminil et al. 2009) are likely to be a result of both the reduced local inbreeding effective population sizes, as well as, particularly, the tendency of selfing species to disperse less pollen (Harder and Barrett 1996; Charlesworth and Pannell 2001), not least because pollen dispersal is usually the dominant mode of gene flow among outcrossing populations (Ennos 1994). However, individuals in the SC population of *L. cavanillesii* do not produce substantially less pollen than those in SI populations (see below), and, as discussed above, are at least as frequently visited by pollinators. It is thus difficult to account for the high level of differentiation between the SC population and the other populations in terms of reduced pollen dispersal. It is more likely that the population lost its diversity as a result of a bottleneck

at the point of colonisation, perhaps by a SC individual, as has been hypothesised for other SC species or populations (e.g. Foxe et al. 2010; Busch et al. 2011).

Perhaps the salient result from our genetic analyses is the extremely high values of genetic differentiation observed among even the SI populations. Here, it is useful to compare our estimates of $F_{ST}$ for *L. cavanillesii* with the distribution found for other species. Figure 5 presents a plot of $F_{ST}$ values and inbreeding levels for 260 species reviewed by Duminil et al. (2009). It is immediately evident that our estimates for *L. cavanillesii* fall among several extreme values. Only four species in Duminil et al.'s (2009) survey show $F_{ST}$ values higher than those for *L. cavanillesii*. Apart from *Caesalpinia echinata*, which is a perennial tree with a high outcrossing rate ($t_m$ = 0.969), these species appear to be typified by highly variable outcrossing rates. *Posidonia australis* (Sinclair et al. 2014) and *Clarkia tembloriensis* (Holtsford and Ellstrand 1992) show some degree of selfing, despite the possession of mechanisms to avoid selfing, and *Eichhornia paniculata* shows variable rates of outcrossing (Barrett and Husband 1990). The variable outcrossing rates in these species are consistent with the fact that they are each likely to be affected by local population bottlenecks as a result of colonization dynamics in a type of metapopulation, with often small demes. The populations of *L. cavanillesii* that we studied were also small, but it is unlikely that they are subject to much population turnover, given the longevity of individual plants (probably > 10 years). Rather, their high values of $F_{ST}$ are probably a direct result of the highly isolated distribution of their specialized habitat, i.e., north-facing limestone cliffs, typically 10s to 100s of km apart, in a highly dissected landscape. The species probably has some features in common with *Antirrhinum valentinum*, also represented in Figure 5, which is a largely SI perennial herb whose populations are small and isolated too (Mateu-Andrés and Segarra-Moragues 2000, 2004).

*Absence of a selfing syndrome*

The loss of SC in angiosperms is often followed by an increase in selfing rates and the evolution of a so-called selfing syndrome, with smaller flowers, and reduced nectar and pollen production (Ornduff 1969;

Cruden 1977; Goodwillie et al. 2010; Sicard and Lenhard 2011). In contrast, we find no evidence for the evolution of such traits in *L. cavanillesii*. Instead, individuals in the SC population have large flowers, and produce copious amounts of nectar, both at levels that were in the upper range for the populations we studied. Similarly, the pollen/ovule ratio found in both SC and SI populations of *L. cavanillesii* (around 600) falls in the range commonly observed for facultative SI species (Figure S5; range 244 to 2599; Cruden 1977).

We can think of at least three possible reasons for the lack of any shift in the SC population of *L. cavanillesii* towards a floral syndrome typical of selfing species. First, it is possible that the loss of SC simply did not lead to predominant self-fertilization because outcrossing has been maintained by selection to avoid inbreeding depression. As we have seen, this does indeed seem to be the case to some extent, as individuals continue to be visited by pollinators and outcross substantial numbers of their offspring. If self-fertilized offspring were doomed to failure as a result of inbreeding depression, the loss of SI would not be expected to prompt a shift to selfing, but rather to the evolution of other mechanisms that ensure outcrossing (Baker 1967). For example, the loss of SI in species of *Lycium* evidently led to the evolution of separate sexes as an outcrossing device (Miller and Venable 2002), as apparently has occurred in many species that lost SI during colonization of oceanic islands following long-distance dispersal (Sakai et al. 1995; Barrett et al. 1996; Pannell et al. 2015). Although it would appear that traits presumably selected for outcrossing in SI *L. cavanillesii* are being maintained in the SC population (large, zygomorphic flowers with nectar spurs containing much nectar), we do not believe this results from selection to avoid inbreeding depression. Our finding of a strong heterozygote deficiency among adults in the SC population ($F_{IS}$ = 0.36) suggests that inbreeding depression is low (Ritland 1990), a hypothesis that is also consistent with direct measures of inbreeding depression (Chapter II).

Second, it is possible that outcrossing is being maintained in the SC population of *L. cavanillesii* to avoid other costs of selfing, such as pollen discounting, i.e., the loss of pollen through selfing that would

otherwise have been destined for outcrossing. Empirical work has shown that pollen discounting can constitute a substantial cost of selfing, particularly in species with large floral displays (Chang and Rausher 1998; Fishman 2000), and theory indicates that it can contribute to the maintenance of outcrossing or mixed mating (Holsinger 1988; Harder and Wilson 1998; Porcher and Lande 2005; Goodwillie et al. 2005). However, again we do not believe that this hypothesis helps to explain the maintenance of an outcrossing syndrome in *L. cavanillesii*. Importantly, the loss of SI will have led to no immediate change in the floral biology of the individuals concerned; they presumably will have continued to produce similar numbers of flowers, with similar amounts of pollen dispersed from anthers placed in the same position beside the stigma. Thus, a recently evolved SC individual should experience no more pollen discounting than a SI individual (unless there were pleiotropic effects of the mutation conferring SC, which also seems unlikely). It is of course possible that SC individuals could have evolved novel floral traits subsequent to the loss of SI, such as subtle changes in phenology, but if pollen discounting constituted an important cost, we should then expect traits to evolve to reduce rather than increase it.

Finally, it is possible that an outcrossing syndrome is observed in SC individuals of *L. cavanillesii* simply because there has been insufficient time for a selfing syndrome to evolve (e.g. Hoebe 2009; Busch et al. 2011). We do not know how long ago SI broke down in *L. cavanillesii*, but it is plausible that the transition to SC was very recent, perhaps too recent for substantial morphological change to have occurred. Immediately following the loss of SI, particularly if it coincided with the colonization of a new population, there would likely have been little genetic variation on which selection could have acted. Because the population may have remained relatively small throughout its (possible young) history, new mutations that might have been beneficial (e.g., by reducing investment in outcrossing) could have been lost easily by drift before being fixed by selection. Ultimately, the extent to which adaptive evolution towards a selfing syndrome occurs (assuming that it is indeed adaptive) must depend not only on how recent the transition to SC was, but also on size of the bottleneck through which the new population was established and on the effective size of the population during its history since the transition. The fact that

many species have evolved a selfing syndrome after a transition to self-fertilization involving a genetic bottleneck suggests either that the bottleneck was not sufficiently severe to deplete variation upon which selection could act, or that sufficient time has elapsed for the accumulation of new adaptive mutations.

Although there is good evidence for the evolution of a selfing syndrome in many species that have lost SI (Lloyd 1965; Ritland 1989; Foxe et al. 2009; Goodwillie et al. 2010), *L. cavanillesii* is by no means the first example of one that has failed to do so. For instance, SI has broken down independently in two different populations of the mustard relative *Leavenworthia alabamica,* in one of which there is little evidence for evolution towards a selfing syndrome, perhaps because the loss of SI was so recent (Busch 2005; Busch et al. 2011). Similarly, SI has broken down in North American populations of *Arabidopsis lyrata*, but this has not been accompanied by important changes in floral morphology, despite increased selfing rates (Mable et al. 2005; Hoebe 2009; Foxe et al. 2010). In *Camissoniopsis cheiranthifolia,* different mating systems can be found in different populations, with some containing large SC flowers associated with mixed mating (Dart et al. 2012), but in contrary to *L. cavanillesii*, inbreeding depression appears quite strong in these populations (Dart and Eckert 2013). It therefore seems that several factors can explain the absence of a selfing syndrome after the loss of SI, with perhaps the most satisfactory for *L. cavanillesii* being a recent transition to SC.

*Concluding remarks*

We were originally prompted to carry out a detailed study of the mating system of *L. cavanillesii* because its large floral displays and apparently massive investment in pollinator reward pointed towards an outcrossing rather than a selfing system, in contrast to what had been claimed in the literature (Carrió et al. 2013). The fact that most of the populations we have now studied do indeed comprise obligate outcrossers provides an illuminating example of how observations of the floral biology and allocation strategies of a plant can inform hypotheses about its mating system before genetic assays or experiments are conducted: we were ultimately not surprised that *L. cavanillesii* is largely an SI outcrosser, after all. Nor is it

surprising that in some of these SI populations a low frequency of individuals exist that show leaky incompatibility and produce some selfing; leaky SI being a common phenomenon in SI species (Levin 1996, e.g. Brennan et al. 2005; Busch 2005; Crawford et al. 2008, 2010; Dart et al. 2012; Zhang et al. 2014). The fact that the inbreeding coefficient for adults in these populations of *L. cavanillesii* is low suggests that inbreeding depression kills selfed offspring before they reach reproductive maturity. This observation, together with the fact that pollinator activity in the populations can be quite high, suggests that selection in these SI populations most likely acts to maintain outcrossing. What does remain a puzzle, however, is that one population has not only lost SI, but now has a mixed mating system with probably low inbreeding depression – it thus joins rank with a great many species with mixed mating systems that have proved enigmatic to explain (Goodwillie et al. 2005). It also remains puzzling that a species that has taken steps on a path towards self-fertilization shows no signs of the selfing syndrome. The hypothesis that the transition to SC was recent is plausible, but it remains somewhat unsatisfactory as an explanation for all the patterns we observe.

## ACKNOWLEDGMENTS

We thank D. Savova Bianchi and J. El-Assad for their help with microsatellites development, L. Liberati and M. Staiff for their help with phenotypic measurements, and N. Ruch for his technical help in the greenhouse as well as with the particle counter. We are grateful to the University of Lausanne for funding the studentship awarded to MV. This work was supported by a studentship from the University of Lausanne.

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**Table 1. Summary of statistical results for the different traits measured in four populations of *Linaria cavanillesii*.** Degrees of freedom (df), F-ratios (F) and p-values are reported for linear mixed models or glm models with binomial data (indicated with a *). Population was treated as a fixed effect. Significant results are highlighted in grey.

| | df | F | p-value |
|---|---|---|---|
| **time to germination** | 3,101.2 | 6.69 | < 0.001 |
| **proportion germination*** | 3,111 | 9.77 | < 0.001 |
| **size after 25 days** | 3,109.1 | 0.26 | n.s (0.26) |
| **size after 16 weeks** | 3,114 | 5.91 | < 0.001 |
| **time to flowering** | 3,115 | 8.4 | < 0.001 |
| **flower production** | 3,110.1 | 2.82 | 0.04 |
| **flower size** | 3,50.6 | 7.81 | < 0.001 |
| **nectar quantity** | 3,56.6 | 4.23 | 0.009 |
| **nectar quality** | 3,37.4 | 3.56 | 0.02 |
| **flower longevity** | 3,60.6 | 1.25 | n.s (0.3) |
| **pollen/ovule** | 3,83 | 0.55 | n.s (0.6) |

**Table 2. Estimates (mean ± sd) of selfing rates, allelic richness, genetic diversity and $F_{IS}$ for the six populations of *Linaria cavanillesii* studied**. SC, SI and SC/SI denote self-compatible, self-incompatible, and mixed populations (with both SI and leaky SI individuals). Selfing rate estimates are based on multilocus heterozygosity data using microsatellites and the software RMES (David et al. 2007). Allelic richness, genetic diversity and $F_{IS}$ were calculated using microsatellites data and the software FSTAT (Goudet 1995). Significant differences among populations for the allelic richness and genetic diversity are indicated with no letter in common. * indicates significant $F_{IS}$ values after Bonferroni corrections.

| Population | selfing rate | allelic richness | genetic diversity | $F_{IS}$ |
|---|---|---|---|---|
| **COV (SC)** | 0.59 ± 0.2 | 1.49 ± 0.5[a] | 0.09 ± 0.13[a] | 0.36* |
| **RUB (SC/SI)** | 0.14 ± 0.16 | 2.12 ± 1.3 | 0.28 ± 0.25 | 0.23* |
| **ZAR (SC/SI)** | 0.15 ± 0.08 | 2.36 ± 1.1 | 0.36 ± 0.27[b] | -0.05 |
| **BUI (SC/SI)** | 0 ± 0 | 2.30 ± 1.3 | 0.31 ± 0.25 | 0.04 |
| **DEN (SI)** | 0.05 ± 0.12 | 2.65 ± 0.7[b] | 0.27 ± 0.21 | 0.05 |
| **BER (SI)** | 0 ± 0 | 2.19 ± 0.9 | 0.34 ± 0.26 [b] | 0.12 |

**Table 3. Pairwise $F_{ST}$ among six populations of *Linaria cavanillesii*.** SC, SI and SC/SI denote self-compatible, self-incompatible, and mixed populations (with both SI and leaky SI individuals). See text for details.

| | COV (SC) | RUB (SC/SI) | ZAR (SC/SI) | BUI (SC/SI) | DEN (SI) | BER (SI) |
|---|---|---|---|---|---|---|
| **COV** | 0 | 0.75 | 0.73 | 0.75 | 0.63 | 0.56 |
| **RUB** | - | 0 | 0.32 | 0.46 | 0.64 | 0.60 |
| **ZAR** | - | - | 0 | 0.28 | 0.60 | 0.53 |
| **BUI** | - | - | - | 0 | 0.63 | 0.55 |
| **DEN** | - | - | - | - | 0 | 0.44 |
| **BER** | - | - | - | - | - | 0 |

**Figure 1. Geographic location of the six different populations of *Linaria cavanillesii* sampled in Spain, with their respective mating systems as revealed by controlled crosses (SC: self-compatible population, SI: self-incompatible population, SC/SI: mixed population containing SI plants and leaky SI plants).**

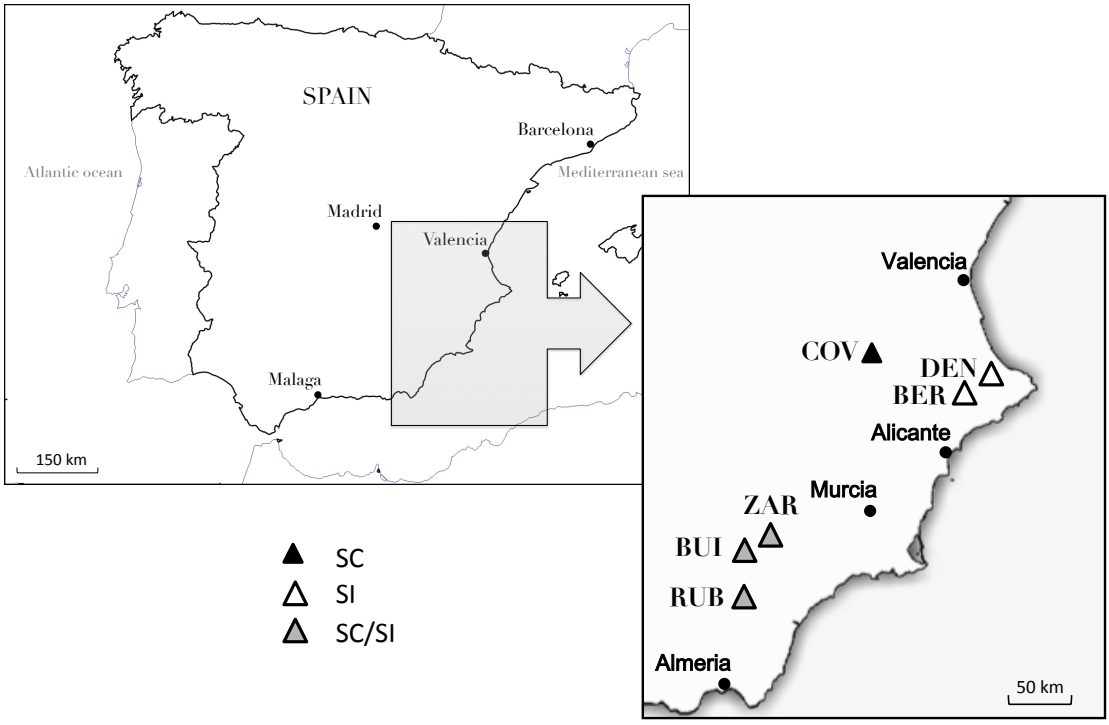


**Figure 2. Comparison of mean seed number produced after controlled crosses of *Linaria cavanillesii* in the greenhouse**. Treatments were performed on twenty plants in each population: autonomous selfing (flower not manipulated); facilitated selfing (pollen coming from the same plant); outcrossing within (pollen coming from another plant within population); and outcrossing between (pollen coming from plants in a different population). In the boxplots, the middle lines represent median, boxes represent first and third quartiles, lower and upper bars represent the minimum and the maximum, and points represent outliers (i.e., points above 1.5 sd).

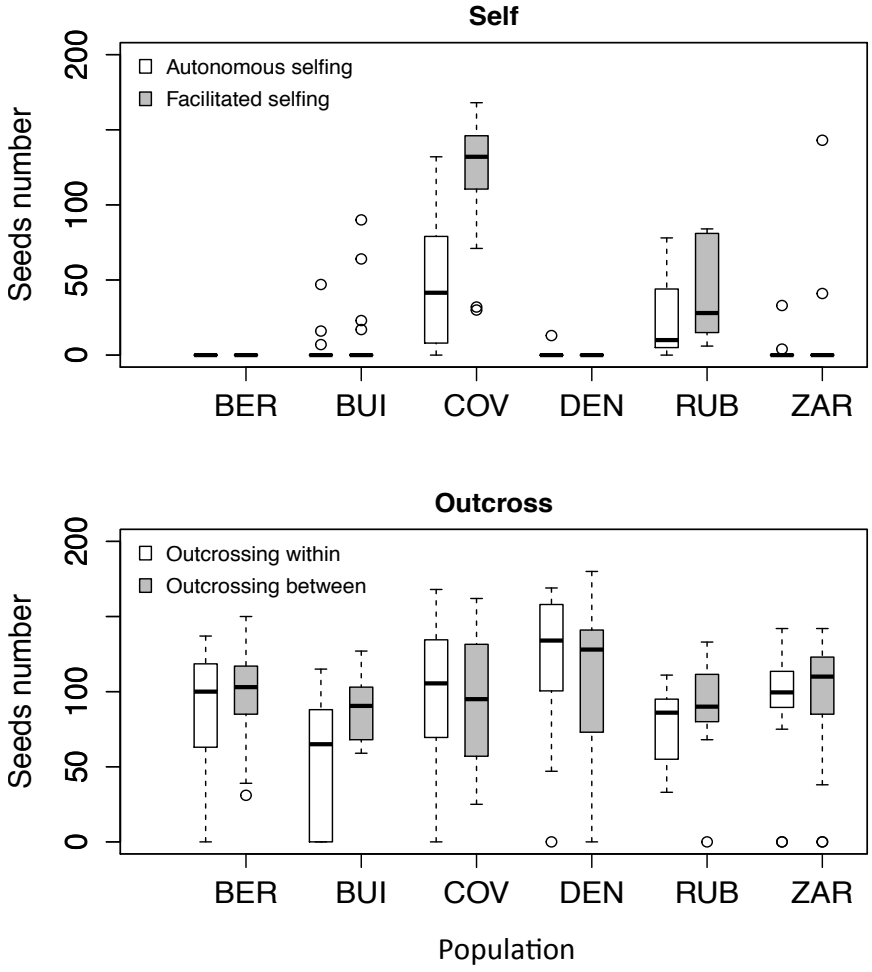


**Figure 3. Estimation of fruit set in six populations of *Linaria cavanillesii* in both 2013 and 2015.** In the boxplots, the middle lines represent median, boxes represent first and third quartiles, lower and upper bars represent the minimum and the maximum, and points represent outliers (i.e., points above 1.5 sd). Diamond symbols represent means for each population. COV: self-compatible population; DEN, BEN: self-incompatible populations, ZAR, RUB, BUI: mixed populations with self-incompatible and leaky self-incompatible individuals.

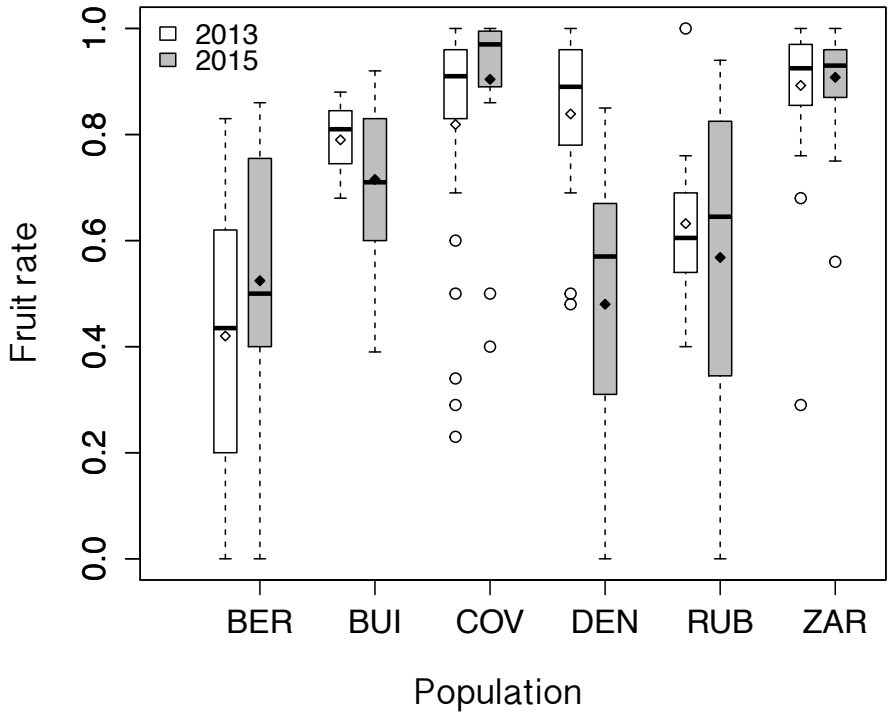


**Figure 4. Comparison of fruit set in six populations of *Linaria cavanillesii* after different treatments**: control bag (inflorescences were bagged at bud stage, and flowers were left unmanipulated); control (inflorescences were left unmanipulated); emasculation (anthers were removed prior to anthesis and the flowers were left unmanipulated) and supplementary outcross pollen (pollen_out: anthers were removed prior to anthesis, and when mature, outcross pollen was applied to stigmas from at least two donor plants from the same population). Populations are SC: self-compatible, SI: self-incompatible, or SC_SI: mixed population containing both type of plants. Bars represent mean +/- SEM, and number of plants used for each treatment is indicated.

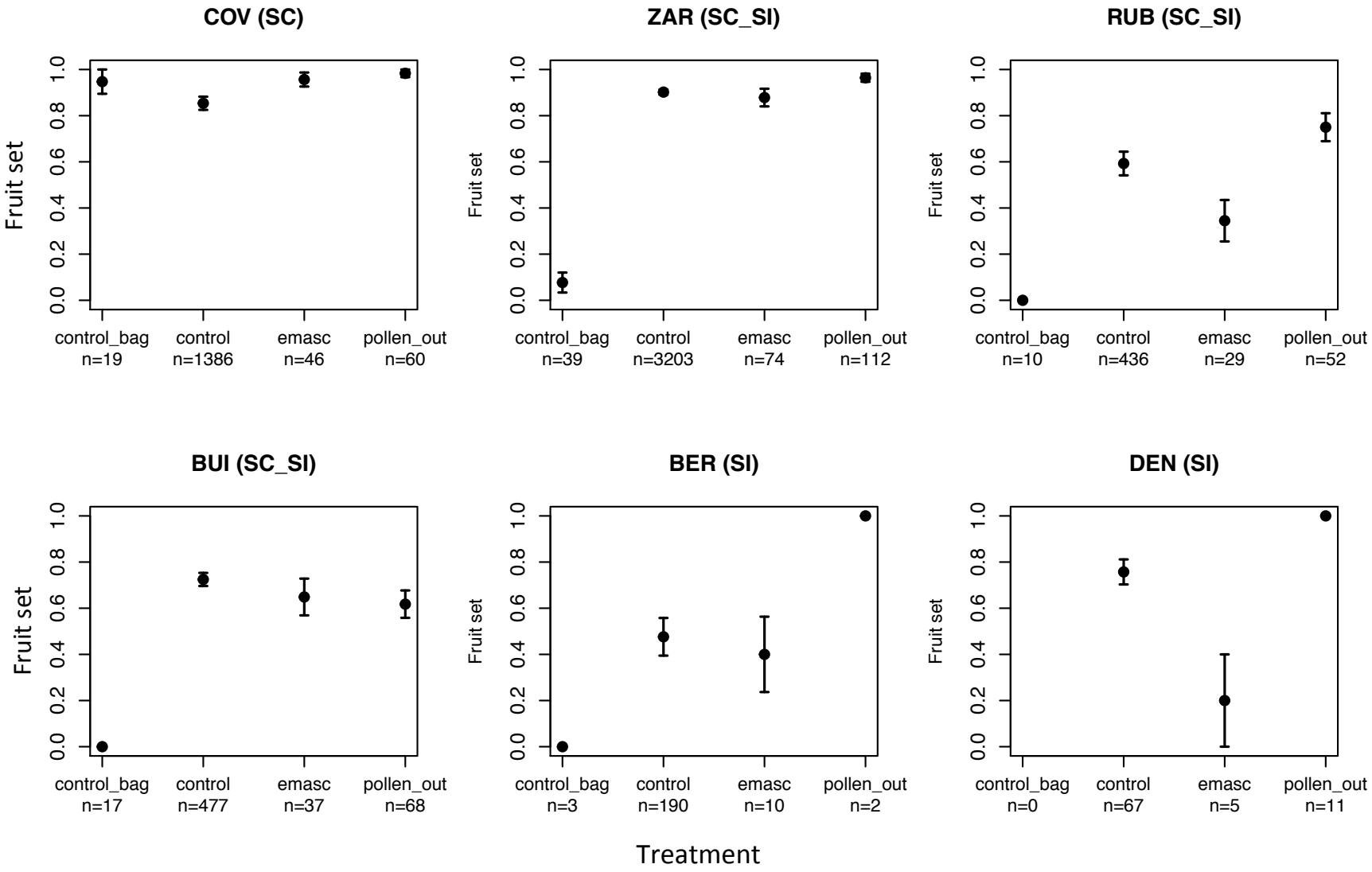
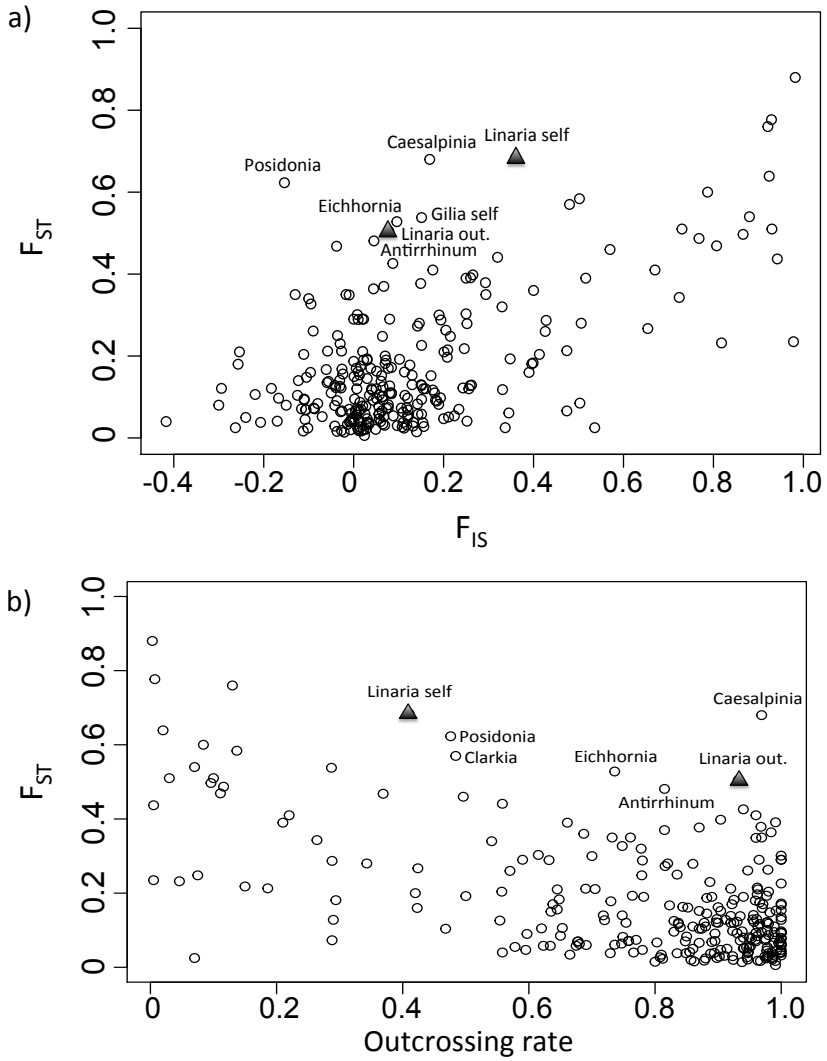


**Figure 5. Correlation between $F_{ST}$ and (a) $F_{IS}$ and (b) estimated outcrossing rates for species reviewed in Duminil et al. (2009).** Estimates for *Linaria cavanillesii,* calculated on the basis of microsatellites data, are shown with black triangles for the selfing and outcrossing populations (labelled "Linaria self" and "Linaria out", respectively). The other species are *Antirrhinum valentinum* (Antirrhinum), *Caesalpinia echinata* (Caesalpinia), *Clarkia tembloriensis* (Clarkia), *Eichhornia paniculata* (Eichhornia), selfing *Gilia achilleifolia* (Gilia self) and *Posidonia australis* (Posidonia). See text for details.



# SUPPLEMENTARY INFORMATION

**Table S1. Names and localizations of the different *Linaria cavanillesii* populations sampled in Spain**

| Populations' Name | Localisations | Populations' size | GPS coordinates (elevation) |
|---|---|---|---|
| Dénia (DEN) | Montgo protected park | ≈ 50 | N 38°49,463' – W 0°06,119' (340m) |
| Bernia (BER) | Sierra de Bernia | ≈ 30 | N 38°39,934' – W 0°03,644' (880m) |
| Cova (COV) | Cova Alta microreserva de flora | ≈ 50 | N 38°48 468 – W 0°30,113' (830m) |
| Buitre (BUI) | Sierra del Buitre | ≈ 100 | N 38°08,423' – W 1°55,261' (1348m) |
| Benizar (ZAR) | Benizar village | ≈ 130 | N 38°15,954' – W 1°59,142' (995m) |
| Rubio (RUB) | Velez-Rubio village | ≈ 30 | N 37°35,162' – W 2°05,155' (1130m) |

**Figure S1. Proportion of fruit formation resulting of controlled crosses of *Linaria cavanillesii* in the greenhouse and depending on populations and treatments.** Population's names are on the x-axis and each treatment has been performed on twenty plants in each population. Bars represent proportion of fruit formed over the 20 crosses. Treatments are: autonomous selfing: flower not manipulated; selfing: pollen coming from the same plant; outcrossing_w: pollen from another plant within population; outcross_b: pollen from another plant between populations.

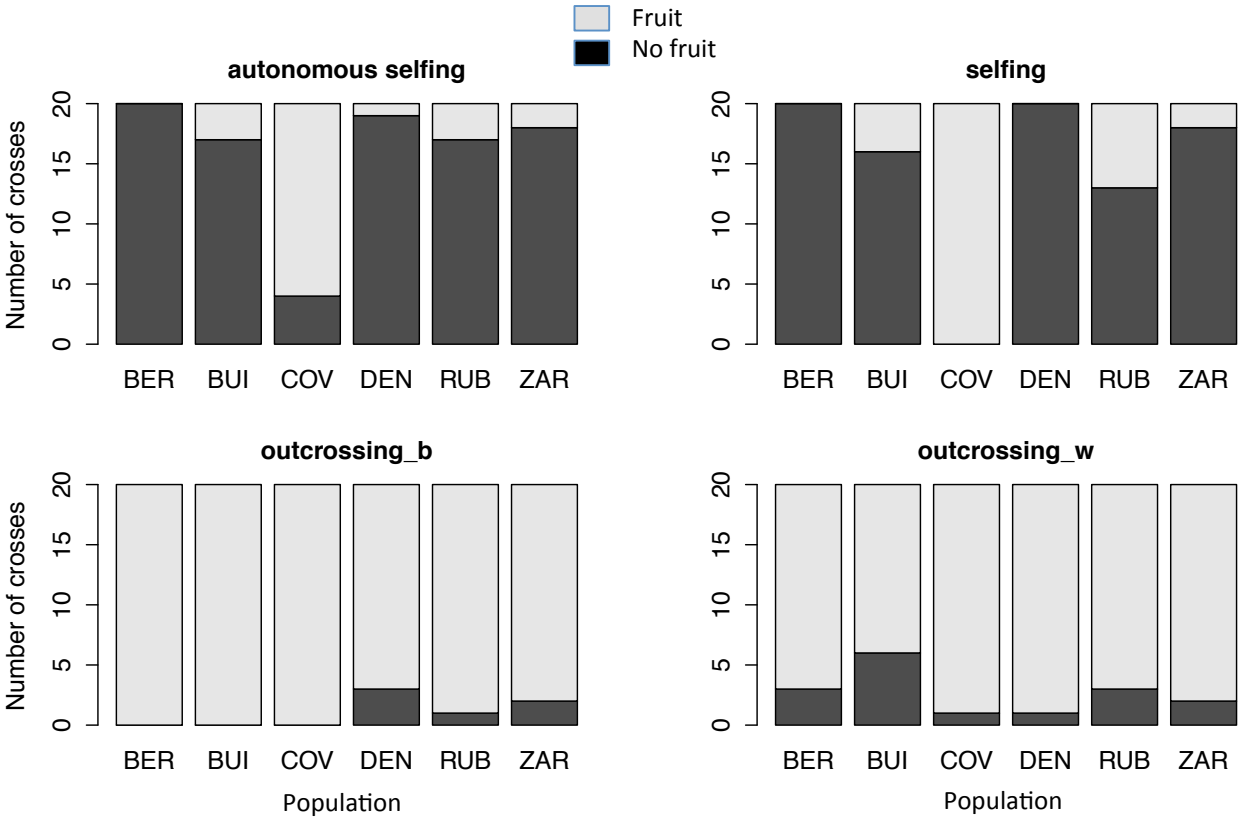


**Figure S2. Comparison of germination time (a) and success (b) for four populations of *Linaria cavanillesii*.** SC: self-compatible population, SI: self-incompatible population, SC/SI: mixed population containing both type of plants; bars represent mean +/- SEM; significant differences from a TukeyHSD are indicated with no letters in common.

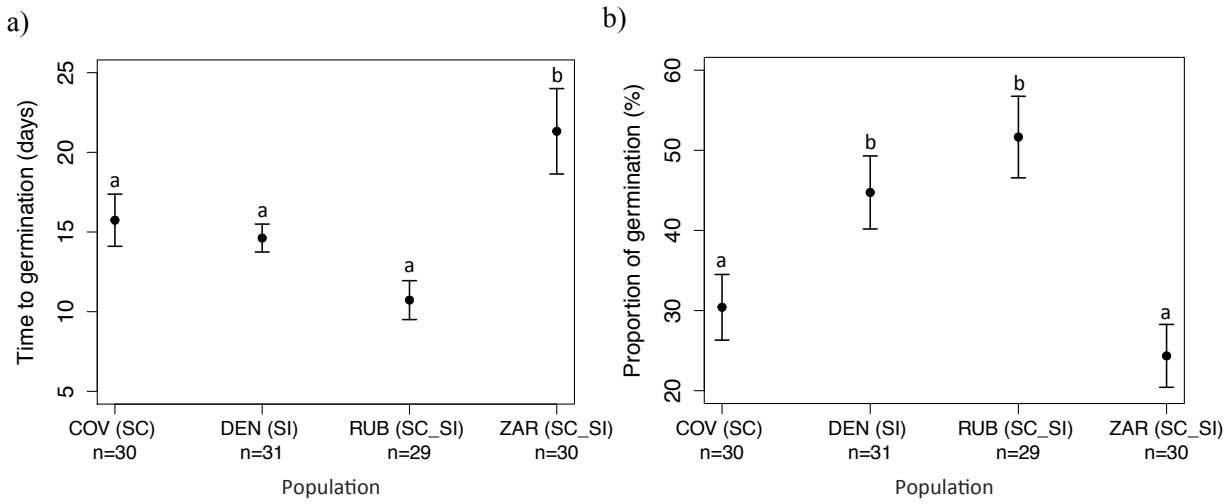


**Figure S3. Comparison of (a) flower longevity, (b) flower size, (c) nectar quantity and (d) nectar quality in four populations of *Linaria cavanillesii*.** SC: self-compatible population, SI: self-incompatible population, SC/SI: mixed population containing both type of plants; bars represent mean +/- SEM; significant differences from a TukeyHSD are indicated with no letters in common.

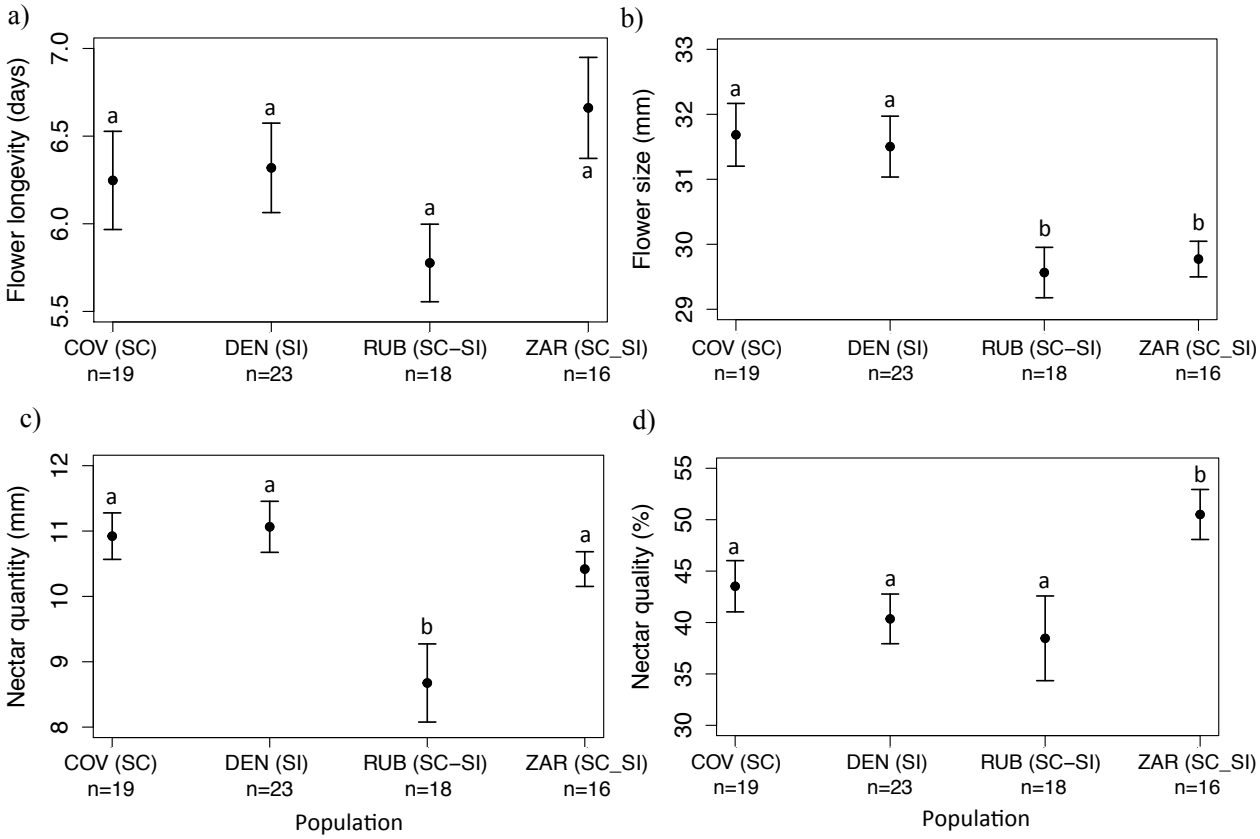


**Figure S4. Comparison of (a) the mean number of days from seeds germination to flowering, and (b) the total number of flower produced over three months in four populations of *Linaria cavanillesii*.** SC: self-compatible population, SI: self-incompatible population, SC/SI: mixed population containing both type of plants; bars represent mean +/- SEM; significant differences from a TukeyHSD are indicated with no letters in common.

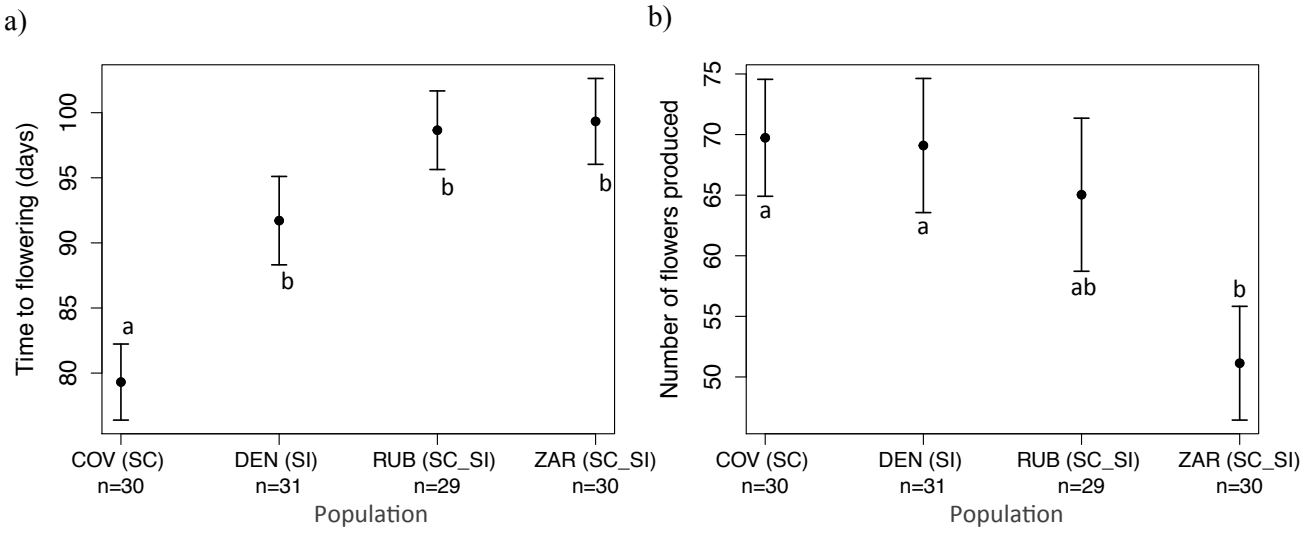
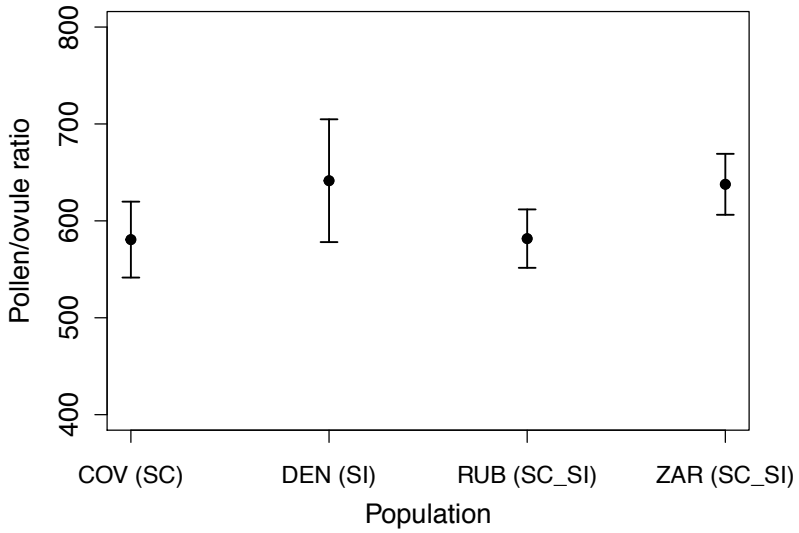


**Figure S5. Comparison of plants pollen/ovule ratio in four populations of *Linaria cavanillesii*.** Each measure was averaged with two anthers per flowers and three flowers per plant. SC: self-compatible population, SI: self-incompatible population, SC/SI: mixed population containing both type of plants; bars represent mean +/- SEM.



**Note S1. Development and characterization of 17 new polymorphic microsatellites markers for the plant species *Linaria cavanillesii***

## Methods and Results

Leave material were collected from six different natural populations of *Linaria cavanillesii* in Southeast of Spain ($29 < n < 41$), and DNA extracted with the DNeasy 96 Plant kit (Quiagen). DNA libraries of microsatellites loci were developed by GenoScreen (France), for a total of 5334 sequences containing microsatellites. Among these, 50 primers were tested for polymorphism. Microsatellites were marked with FAM, HEX or ATTO colors (Microsynth), and amplifications were carried out by PCR in a Biometra thermocycler. The amplification reaction contained 1xPCR mix: 2 ng/µL template DNA, 10x PCR Buffer, 25 mM $MgCl_2$, 5x Q-solution, 2.5 mM dNTP, 0.2 µM of each primer and 0.5 U/µL of Taq DNA polymerase (HotStarTaq®, Qiagen). The thermocycling conditions were 15 min at 95 °C followed by 32 cycles of 30 sec at the annealing temperature (Table1), 30 sec at 72 °C and 30 sec at 95 °C, followed by one cycle of 1 min at the annealing temperature (Table1) and final extension of 30 min at 72 °C. PCR products were then sequenced in an ABI3100 (Applied Biosystems). Microsatellites data were analyzed with GeneMapper®. A total of 17 primer pairs were polymorphic, with 2 to 12 alleles per locus (Table 1). Finally, the program Fstat (Goudet, 1995) was used to calculate expected and observed heterozygosity for each loci.

**Table 1- Summary of characteristics for 17 polymorphic markers isolated from *Linaria cavanillesii***

| Name | Locus | Repeat type | Sequence (5'-3') | T° | size range (bp) | Ho | He | nb of alleles |
|---|---|---|---|---|---|---|---|---|
| 1 | LIN1 | (ACA)5 | ACA GTG ACC GAT TTA TCC CC<br>CGA AGA GTG CAG CTT TGA GA | 58 | 126-135 | 0.305 | 0.288 | 4 |
| 4 | LIN4 | (GTT)5 | ACC TGC TTC GTC AGC TTT TC<br>CTG GCA AGA ACT TGA GAG GG | 56 | 128-135 | 0.308 | 0.284 | 3 |
| 6 | LIN6 | (CAACA)7 | AAT TTT AGC CGA ATT AAA CAA AAG<br>CAT GTT CTT GGG GTT CCA TT | 56 | 211-238 | | | 8 |
| 7 | LIN7 | (CCAAT)5 | GCA GAA ATT TTC GAA TGC CT<br>CTG GTT CGT TTG GTC CTG TT | 58 | 152-182 | 0.356 | 0.44 | 8 |
| 15 | LIN15 | (TG)9 | ACC AAA CAA CAC ACA TGG CA<br>TGC CAG CTT TCA TGA GCA TA | 53 | 127-135 | 0.289 | 0.358 | 6 |
| 19 | LIN19 | (GAACAC)5 | CCT TCC ATT TCG AAT TAA CAT C<br>ATT GGA GAG GAT TCC GCC | 53 | 173-190 | 0.198 | 0.205 | 4 |
| 21 | LIN21 | (AG)5 | TGA CAG CTG ACC TGA CGA TT<br>ACT CGT CTC CGT AAC CAC CA | 57 | 107-109 | 0.057 | 0.062 | 2 |
| 28 | LIN28 | (CAA)6 | CAC CTT TCC CGA TCC TTG TT<br>CTA GGG TTT CGG TTC GGA TT | 55.5 | 116-132 | 0.269 | 0.363 | 4 |
| 31 | LIN31 | (TC)9 | CAC AAC TCG CTT TTC GTC CT<br>TTG CAA CGG TCG AAT TTA TG | 53 | 107-129 | 0.618 | 0.574 | 11 |
| 32 | LIN32 | (GTG)6 | ATG TGA CAC GTT CAC TGC GT<br>GTC GGA TAC CGT GAC CTG AT | 57 | 154-169 | 0.145 | 0.176 | 5 |
| 35 | LIN35 | (TTG)6 | TTC AAA CTC GCC AAA TCA TGT<br>CAG AGC ACA GAG TTG TCA TCG | 54.5 | 122-131 | 0.105 | 0.259 | 4 |
| 37 | LIN37 | (AGA)5 | CGA CAT TGA TCG AAA GGA AT<br>TTC AAC ATT TTG TTT CTT CTT CTC T | 53 | 98-104 | 0.153 | 0.146 | 3 |
| 39 | LIN39 | (CTC)5 | GTG CGA GAA GTT TCT AAT ATC TAG TTG<br>AAG AAA TGC AAA ATT GGA ATG TG | 53.5 | 155-191 | 0.137 | 0.165 | 3 |
| 45 | LIN45 | (TCT)5 | AAT CTC CGT GTT CGC GAT T<br>TCA CTT TGT GAC GCT TTT GC | 53 | 162-180 | 0.214 | 0.205 | 8 |
| 46 | LIN46 | (TG)10 | CAT GGA GGT GTT AGC CTC CG<br>TCA AAA CAC GCT TTA TCG CC | 57 | 180-204 | 0.446 | 0.449 | 12 |
| 50 | LIN50 | (TCC)5 | AAC AGG GTC TGG CAA ACA TT<br>AGA ATT GGG AGG ATG GAG GT | 54.5 | 119-125 | 0.166 | 0.168 | 3 |
| 51 | LIN51 | (CA)12 | GCT TAA ATC CAC AAC GCC A<br>GGT GTA ACT GAA TTG TAT GTA TAG GTG | 54.5 | 149-165 | 0.255 | 0.266 | 7 |

**$T_a$, annealing temperature of the primer pairs; $H_E$, expected heterozygosity; $H_O$, observed heterozygosity.**

# - CHAPTER II -

## Inbreeding depression is high in a self-incompatible perennial herb population but absent in a self-compatible population showing mixed mating

M. Voillemot and J. R. Pannell

*(Submitted in Heredity)*

# ABSTRACT

High inbreeding depression is thought to be one of the major factors preventing evolutionary transitions in hermaphroditic plants from self-incompatibility (SI) and outcrossing toward self-compatibility (SC) and selfing. However, when selfing does evolve, inbreeding depression can be quickly purged, allowing the evolution of complete self-fertilization. In contrast, populations that show intermediate selfing rates (a mixed mating system) typically show levels of inbreeding depression similar to those in outcrossing species, suggesting that selection against inbreeding might be responsible for preventing the transition towards complete self-fertilization. Here, we compared levels of inbreeding depression between populations of *Linaria cavanillesii* (Plantaginaceae), a perennial herb showing contrasting mating systems. The SI population showed high inbreeding depression, whereas the SC population displaying mixed mating showed no inbreeding depression. In contrast, we found that heterosis based on between-population crosses was similar for SI and SC populations. Our results are consistent with the rapid purging of inbreeding depression in the derived SC population. However, the maintenance of substantial outcrossing is inconsistent with the expectation that populations that purged of their inbreeding depression should evolve towards complete selfing. Our study provides further evidence that the loss of SI in *L. cavanillesii* has been very recent.

**Keywords:** genetic load, heterosis, mating system, purging, outcrossing, selfing

# INTRODUCTION

Hermaphrodites can potentially reproduce via a mix of self-fertilization and outcrossing. Although self-fertilization allows plants to transmit two copies of their genome to their seed progeny rather than only one, with a substantial potential fitness advantage (Fisher 1941), the expression of inbreeding depression by selfed progeny (reduced fitness of selfed compared to outcrossed individuals) is thought to constrain the evolution of selfing in hermaphrodite individuals (Charlesworth and Charlesworth 1987; Winn et al. 2011). Selfing is expected to decrease heterozygosity, resulting in the expression of deleterious recessives alleles and an increase in inbreeding depression. Although selection against inbreeding and the effects of inbreeding depression have likely contributed to the maintenance of various mechanisms to ensure outcrossing, not least self-incompatibility (e.g. Sletvold *et al.*, 2013), numerous formally self-incompatible (SI) species have undergone evolutionary transitions to self-compatibility (SC) and decreased outcrossing rates (Goodwillie et al. 2005). These transitions may have been driven by the selective advantage gained by selfing individuals through the transmission of more genes to their seed progeny through both their ovules and the self pollen grains that sire them (automatic transmission advantage; Fisher, 1941) as well as through an ability to reproduce by in the absence of mates or pollinators (reproductive assurance advantage; Jain 1976).

Whatever its cause, a transition from SI to SC and increased self-fertilization should bring about an elevated homozygosity in the population and thus the expression of recessive deleterious alleles that would otherwise be protected from purifying selection in heterozygotes. As a result, mutations that cause inbreeding depression can be quickly purged from the population (Dart and Eckert 2013; Noël et al. 2016). With inbreeding depression purged, selection now should more strongly favour the maintenance of self-fertilization (Lande and Schemske 1985) and traits that increase its rate and efficiency (Goodwillie et al. 2010). Accordingly, not only do populations that have undergone an evolutionary transition to selfing tend to show substantially reduced inbreeding depression (Husband and Schemske, 1996), but they

typically also have smaller flowers than their outcrossing progenitors, with reduced pollen:ovule ratios and reduced nectar production, i.e., they often display a 'selfing syndrome' (Goodwillie et al. 2010).

Intriguingly, not all species that lose an SI system and acquire a capacity to self-fertilize undergo a transition to full (or nearly full) self-fertilization and a selfing syndrome. Indeed, many SC species that are derived from SI ancestors continue to outcross to a substantial extent (i.e., they display 'mixed mating'; and they continue to maintain floral traits in common with outcrossers, with large flowers, copious nectar production, and high pollen:ovule ratios (Fenster and Martén‐Rodríguez 2007; Dart et al. 2012). Indeed, of 345 flowering plants species surveyed by Goodwillie et al. (2005), about 40% were estimated to have intermediate selfing rates between 0.2 and 0.8. Although there are numerous models that can explain the maintenance of mixed mating, via a number of different mechanisms, mixed mating systems continue to represent an enigma (Goodwillie et al. 2005).

The enigma of mixed mating pertains not least to patterns of inbreeding depression. One might expect that intermediate selfing rates of species with a mixed mating system should bring about the purging of inbreeding depression and thus a selected shift towards greater selfing. However, in a recent survey of the literature, Winn *et al.* (2011) found that species with mixed mating systems show levels of inbreeding depression similar to those of outcrossing species. Although they found lower values of inbreeding depression for selfing compared to outcrossing taxa, consistent with the theoretical expectation of purging (Husband and Schemske 1996), they also found that inbreeding depression in mixed-mating populations was similar to that found in fully outcrossing ones. Mixed mating might therefore be interpretable as the result of selection to maintain outcrossing in species with a high load of deleterious recessive mutations but in which some selfing is an unavoidable consequence of the pollination mode (e.g. Kalisz *et al.*, 2012; Dart and Eckert, 2013).

In addition to its effects on inbreeding depression, a transition to increased selfing might also affect patterns of heterosis (the increased fitness of offspring resulting from between-population crosses compared to crosses within populations). Indeed, even populations that have purged their inbreeding depression might still maintain substantial genetic load as a result of the fixation of mildly deleterious recessive mutations during a population bottleneck associated with the mating-system transition (Kirkpatrick and Jarne 2000), or simply because the effective population size is diminished by increased inbreeding (Roze and Rousset 2004). Moreover, populations that have recently shifted to SC are usually associated with isolation, reduced size and/or increased population differentiation (Duminil et al. 2009), which can all bring about increased heterosis in between population crosses. Such patterns have been found in a number of studies. For instance, Busch (2006) did not find heterosis among five large SI populations and two small SC populations of *Leavenworthia alabamica*, but found high levels of heterosis expressed in crosses involving the most isolated self-fertilizing population. In another recent study, Oakley and Winn (2012) found greater heterosis for small compared to large populations (see also Escobar et al. 2008).

Here, we assess levels of inbreeding depression and patterns of between-population heterosis for one leaky outcrossing SI and one mixed-mating SC population of the long-lived perennial herb, *Linaria cavanillesii,* which shows variation in self-incompatibility and its mating system, with populations either fully SI, partially SI (SI individuals mixed with leaky SI individuals) or fully SC (Chapter I). Interestingly, despite its capacity for autonomous self-fertilization, the one known SC population maintains a high rate of outcrossing (selfing rate = 0.59) and displays floral traits comparable to that in the SI populations (large floral displays, high pollen:ovule ratios, and high nectar production), suggesting a possible recent transition to SC (Chapter I). Populations or *L. cavanillesii* show clear evidence for strong isolation by distance (and thus for stepping-stone migration), with the strongest differentiation observed between the SC and the other populations (Figure 1). Although we have found only one SC population of *L. cavanillesii*, observed patterns of inbreeding and inbreeding depression nevertheless help to broaden our

inference base for understanding the transitions between mating systems in plants, not least because *L. cavanillesii* is an outlier in the relation between inbreeding and population isolation (Chapter I; and see Discussion).

We expected to find high inbreeding depression maintained under SI and obligate outcrossing. Moreover, because the SC population does not appear to have undergone a transition towards a selfing syndrome and maintains intermediate to high outcrossing rates, we expected to find high inbreeding depression in this population, too, in line with patterns commonly observed for mixed-mating species (Winn et al. 2011). Finally, we predicted higher heterosis for crosses involving the SC population compared to SI ones, e.g., due to the possible fixation of mildly deleterious alleles that may have occurred following a population bottleneck associated with the breakdown of SI and the reduced effective population size under partial inbreeding. Surprisingly, although we found substantial inbreeding depression under SI and patterns of heterosis that were largely consistent with our expectations, there was little evidence for any inbreeding depression in the SC population.

# MATERIAL AND METHODS

*Site and study species*

*Linaria cavanillesii* is a perennial herb, endemic to southeastern Spain, that occurs along north-northwest-oriented cliffs at elevations of between 300 m and 1400 m. Flowering occurs between May and June, during which yellow nectar-spurred flowers are held in large inflorescences that are attractive to pollinators, mainly bees and bumblebees. Around 30 days after fertilization, seeds are dispersed passively from capsules to the wind. See Chapter I for further details. This study was carried out using seeds from three different populations in the Alicante-Murcia region: one fully SC and one leaky SI population as maternal plants; and one fully SI population for additional pollen donors (Figure 1a).

*Hand-pollination crosses*

To assess and compare inbreeding depression as well as levels of heterosis between one SC and one SI population, we performed controlled crosses in the greenhouses of the University of Lausanne, Switzerland. We used two populations of *Linaria cavanillesii* as maternal plants: the fully SC population with mixed mating ($COV_{(SC)}$); and a leaky SI population ($BUI_{(SI)}$) which comprise mostly SI individuals as well as some individuals with leaky SI (low production of selfed seeds). One difficulty faced when assessing inbreeding depression for most outcrossing taxa is the difficulty of procuring selfed seed from SI individuals. In *L. cavanillesii,* as in many SI species (e.g. Crawford et al. 2010; Dart et al. 2012; Zhang et al. 2014), the occasional production of selfed seeds is common in some populations, a phenomenon known as pseudo-self-compatibility or leaky SI (Levin 1996). In this study, we obtained selfed progeny from seeds produced via pseudo-self-compatibility for the SI population. Additionally, one other SI population ($DEN_{(SI)}$) was used as donor plants to compare levels of heterosis (see Figure 1a).

We applied four different treatments: 'self-pollination' (inflorescence bagged at the bud stage, anthers removed prior to anthesis, and pollination with self pollen); 'outcrossing within population' (inflorescence bagged at the bud stage, anthers removed prior to anthesis, and pollination with outcross pollen from a different plant of the same population); and two 'outcrossing between populations' (inflorescence bagged at bud stage, anthers removed prior to anthesis and fertilization with outcross pollen from a plant coming from a different population, either $DEN_{(SI)}$, $BUI_{(SI)}$ or $COV_{(SC)}$, depending on the treatment). When describing crosses, the first population name represents the maternal plant, and the second one the pollen donor. Crosses between populations were used to compare: (1) heterosis between distant populations and SC vs. SI populations (crosses $COV_{(SC)}$-$BUI_{(SI)}$ and $BUI_{(SI)}$-$DEN_{(SI)}$ respectively); (2) heterosis between distant and nearby populations for the SC population (crosses $COV_{(SC)}$-$BUI_{(SI)}$ and $COV_{(SC)}$-$DEN_{(SI)}$, respectively); and (3) maternal effects on heterosis (reciprocal crosses $COV_{(SC)}$-$BUI_{(SI)}$ and $BUI_{(SI)}$-$COV_{(SC)}$).

The data we present are derived from a total of 82 crosses using nine and thirteen mother plants from the $BUI_{(SI)}$ (40 crosses) and $COV_{(SC)}$ populations (42 crosses), respectively, and our results are based on fitness traits measured on a total of 208 progeny plants raised to maturity (98 for $COV_{(SC)}$ and 110 for $BUI_{(SI)}$; see Table 1 for details). Our sampling was limited by difficulties we faced in raising plants from seeds (see Discussion), but our analysis nevertheless allows us to draw several clear conclusions.

To exclude pollinators, we bagged inflorescences with small fine-meshed nylon bags a few days before flowers opened, and we marked individual flowers with colored string corresponding to each treatment. We removed anthers carefully with fine forceps at the same time. After three days (when stigmas were receptive), we applied appropriate pollen, depending on treatment. Self-pollen was taken from intact flowers on the same plant, whereas outcross pollen came from another plant from the same or from a different population. We applied pollen by gently brushing an anther against a target stigma until it appeared fully covered with pollen. After 14 days, we investigated any successful fertilization by observing fruit formation. We applied rubber glue to the apex of developing fruit capsules after 18 days, when fruits were fully formed, to prevent seed loss before collection. We collected fruits approximately ten days after they had been glued to ensure that seeds were fully mature.

*Progeny phenotypic and fitness measurements*

To estimate different phenotypic and fitness-related traits, we counted and weighed all seeds for each cross. We then sowed ten seeds from each fruit in Petri dishes placed in a phytotron (conditions: 13 hours days, 20° C:15° C day:night, 80% humidity) and assessed germination rate, accordingly. We then transplanted four randomly chosen seedlings into larger pots, noting seedling size at the time of transplantation. All plants were placed on glasshouse benches in a random block design. Additionally, we moved each table and randomized plants within the table once a week. We measured growth after nine weeks, and recorded the number of days from transplantation to flowering, as well as flower production, over a period of three months. We also measured phenotypic traits, including flower longevity, flower

size, pollen production, ovule number, pollen:ovule ratio, and nectar quantity and quality (measured with two refractometers to cover full range of nectar concentration: 0-50 Brix; and 45-80 Brix; Bellingham & Stanley Ltd, Tunbridge Wells, UK). To account for flower variability within plants, we took and averaged every measure of flowers using at least three flowers per plant.

We used a particle counter (Elzone II 5390 Micromeritics®) to estimate the number of pollen grains. For each sample, one upper and one lower anther of a non-opened flower were fixed in formaldehyde-acetic acid alcohol solution (FAA; 5 parts glacial acetic acid: 5 parts 38% formaldehyde: 90 parts 70% ethanol). Before analysis, samples were sonicated (Branson 52, Emerson industrial automation) for two minutes to release pollen from anthers, and then transferred into the analysis beaker of the particle counter, which contained 100 mL of $ddH_2O$ with 2% NaCl. Each sample was analysed four times for thirty seconds, and the average of the four replicates was used to estimate the number of pollen grains per anther. We estimated pollen production for each plant as the average over measures taken for three flowers per plant. We estimated the number of ovules for the same flowers used for pollen counting, based on floral dissections using a razor blade, and counted under a binocular microscope (Leica MZ 125, Leica Microsystems ®).

*Data analysis*

All analysis was conducted in R (version 3.1.2, R Core Team 2015). We analyzed seed production and seed mass resulting from initial crosses using analysis of variance, using data on per-family averages, and using Tukey post-hoc tests for multiple comparison of means. For most other traits, we analyzed the effect of crosses for each population with linear mixed models, using the lme4 package in R (Bates et al. 2014), and setting pollination treatment as a fixed factor and block and mother as nested random factors. For the assessment of phenotypic differences, we included the timing of measurement as an additional random factor. For nectar measurements, we included temperature and humidity as random factors. We analyzed significance of variables through a stepwise deletion procedure; in the final model, multiple comparisons

of means were performed using the lmerTest package in R (Kuznetsova et al. 2014). For binomial data such as the proportion of seed germination, we used a generalized mixed model (glmer), with maternal plant identity treated as random factor and data modeled as binomial. If significant, differences within treatments were then tested by means of post-hoc tests, with the glht function of the multcomp package in R. All statistical results are summarized in Table 2.

*Estimates of heterosis and inbreeding depression*

Following Ågren and Schemske (1993), inbreeding depression was calculated as $1 – (w_s / w_{ow})$ when selfed offspring has lower trait values than outcrossed progeny, and as $(w_{ow} / w_s) - 1$ otherwise; where $w_s$ and $w_{ow}$ are per-family mean trait values in selfed and outcrossed within population treatments, respectively. Values of inbreeding depression were thus scored on a scale from -1.0 to 1.0, with larger values indicating higher levels of performance, except for time to flowering, which we inversed for interpretation. Heterosis was calculated as $1- (w_{ow}/w_{ob})$, where $w_{ow}$ and $w_{ob}$ are per-family mean trait values in outcrossed within population and outcrossed between population treatments, respectively. Positive heterosis indicates higher levels of performance of progeny resulting from between-population crosses, whereas negative heterosis indicates outbreeding depression.

Many traits measured are phenotypic traits (e.g. nectar production), but some traits are more directly related to fitness. Thus to estimate a composite measure of early-acting inbreeding depression based on fitness-related traits, we computed an average lifetime cumulative index of fitness based on the product of seed production (but see Discussion), proportion of seed germination, plant growth, number of flowers produced at the time of harvest and estimate of the number of pollen grains and ovules produced per flower. Because *L. cavanillesii* is a long-lived perennial herb that probably lives for many years, it was not possible to estimate components of inbreeding depression expressed in older adult plants (see Discussion).

# RESULTS

*Measures of inbreeding depression*

Considering all the 13 variables investigated, inbreeding depression (lower value for selfed vs. outcrossed progeny) for the SC population was significant only for nectar quality (Table 2; Figure 2; Figures S1-S7). In contrast, the SI population showed significant inbreeding depression for four of the traits measured (seed production, days to flowering, growth after nine weeks and flower size; Table 2; Figure 2; Figures S1-S7). The average fitness index gave an inbreeding depression value of 0.00 for the SC population and 0.85 (including measures on seed production) or 0.49 (excluding seed production; see Discussion) for the SI population (Figure 2).

*Measures of heterosis*

Our results revealed heterosis for both the SI and the SC populations (Figure 3, Figures S1-S7). Except for seed production, every trait measured in the SC population showed a greater fitness when plants were crossed with plants from another more distant population (Figure 3). For the SI population, only time to flowering and nectar quality gave a lower fitness (Figure 3). The average lifetime fitness index gave a heterosis value of 0.33 for the SC population and 0.27 for the SI population.

For crosses involving the SC population, we found a lower heterosis when crosses were performed with the nearby population (Figure 3). Among the 13 traits measured, only flower size and pollen production showed significant heterosis for the crosses with a nearby population (Table 2; Figure 3, Figure S4 and S6). The average fitness index yielded a heterosis value of 0.08 compared to 0.33 for distant population crosses. Finally, reciprocal crosses of SC and SI populations showed strong maternal effects, with heterosis being 0.33 when the pollen donor was SI, and -0.32 when the pollen donor was SC (Figure 3).

# DISCUSSION

As expected for an outcrossing perennial population (Winn et al. 2011), our study revealed high inbreeding depression (ID) for self-fertilized individuals from a self-incompatible (SI) population of *Linaria cavanillesii* (for days to flowering, growth, flower production, and flower size). In contrast, we found no evidence for ID for crosses of a self-compatible (SC) population that displays mixed mating. In both populations, substantial heterosis was found for crosses between more distant populations, but crosses between nearby populations involving the SC population resulted in a lower heterosis. Finally, we found evidence for strong maternal effects on heterosis, with the results of SC vs. SI crosses depending on which population contributed ovules vs. pollen. Our study involved comparisons with the only fully SC population of L. cavanillesii that we have been able to find, and thus might seem to represent a narrow base for inference. However, because the evolution of selfing must usually begin in a single population, sampling other populations into which it has later spread would not increase the inference base in any meaningful way. Our results are based on crosses involving the single known transition to SC in L. cavanillesii and are likely informative about the very early stages of the potential spread of a capacity to self. They also reveal the outcome of a breakdown in SI in a plant that occupies an extreme position among plants in the relation between inbreeding and population isolation and thus help to broaden the base of inference for understanding the genetic consequences of mating-system shifts in plants.

*High inbreeding depression in a self-incompatible population*

Intra-population crosses revealed significant ID for the SI population, with the traits most affected mainly concerning time to flowering and flower production. We also found that selfing yielded lower number of seeds than outcrossing. Because we estimated seed production on the basis of the spontaneous production of seeds by leaky SI individuals, this result is likely to be partly due to poor fertilization rather than ID. Nevertheless, the presence of aborted seeds in selfed fruits suggests that ID may also have affected early seed development, too. In what follows, we take a conservative view and ignore possible effects of ID

during this early stage of the plant's life cycle. Calculating a combined index of ID across all stages measured, ignoring seed set, we estimate that ID for outcrossing populations of *L. cavanillesii* is $\delta = 0.49$. If the difference in seed set between selfed and outcrossed progeny were fully attributed ID, this estimate would increase substantially to $\delta = 0.85$. It seems likely that $\delta$ for outcrossing populations of *L. cavanillesii* lies between these two values, not least because we measured components of fitness for only young plants in a perennial species in which individuals may live for many years. It is thus plausible that $\delta > 0.5$ for outcrossing populations of *L. cavanillesii*. An estimate of $\delta > 0.5$ is consistent with a scenario in which outcrossing should be maintained by selection (Lande and Schemske 1985), and it is in broad agreement with empirical studies; e.g., in a review of estimates of ID for 58 outcrossing plant species, Winn et al. (2011) calculated an average lifetime of $\delta = 0.54$. More recently, a study on the perennial *Arabidopsis lyrata* revealed that two of its SI populations showed high ID (Sletvold et al. 2013). It would thus seem that ID may be an important force preventing the selective loss SI or the spread of SC in populations of *L. cavanillesii*. Even if ID is not the only force that can constrain a shift to selfing (an additional force might be pollen discounting; Porcher and Lande, 2005), the fact that almost all populations of *L. cavanillesii* surveyed had a low proportion of individuals with leaky SI (partial SC) still suggests that SC could have spread if it was advantageous.

*Low inbreeding depression in a mixed-mating population*

We found an absence of ID in the SC mixed mating population, whether measured at early or late stages of growth (average cumulative $\delta = 0.0$). Recall that this mixed mating population shows relatively high levels of outcrossing (selfing rate = 0.59, Chapter I). The absence of ID is thus somewhat surprising, as mixed mating species tend to harbor similar levels of ID as SI species (Winn et al. 2011). Rather, our result is more in line with expectations for highly selfing species, in which ID is quickly purged (e.g. Busch, 2005; Noël *et al.*, 2016). The observed patterns of mating and lack of ID in the SC population of *L. cavanillesii* thus pose the intriguing question as to why outcrossing persists in the face of the expected automatic selection of increased selfing (Fisher 1941).

Ours is not the first study to have found a lack of ID in a plant species with mixed mating. In a comparison of ID in species with different mating systems, Goodwillie *et al.* (2005) found that of 64 species showing mixed mating (i.e., 0.2 < selfing rate < 0.8), ID was positive but < 0.5 in ten species and was negative in seven species. Even if some of these species may be in a state of evolutionary transition toward higher selfing rates (e.g. Dart and Eckert, 2013), it is still not clear what might stabilize others.

Several hypotheses are suggested to explain how mixed mating could be stable. For example, stability can result from frequency dependent pollination processes. In its models, Holsinger (1991) has shown that pollen discounting can maintain mixed mating, predicting that in a highly selfing population, an individual exporting pollen would always have a transmission advantage, therefore allowing mixed mating to become stable. This has been observed in Ipomonea purpurea for instance, where frequency dependent pollen discounting has indeed been shown to maintain mixed mating (Chang and Rausher 1998). Later on, this result was confirmed by more complicated models including variation in ID and pollen limitation, even if low stable intermediate selfing rates were also shown to result from unavoidable geitonogamy (Porcher and Lande 2005a). Additionally, mixed mating can be stable if it provides reproductive assurance in cases of spatiotemporal variation in pollinator services. In Collinsia verna, for instance, variability in pollinator environment seem to be sufficient to maintain substantial outcrossing rates despite low ID (Kalisz and Vogler 2003, 2004). However, in most cases, the absence of shift toward high selfing in species showing an absence of ID still remains puzzling and unexplained.

We can advance three possible explanations for the combination of mixed mating and the absence of ID observed in the SC population of *L. cavanillesii*. First, if the loss of SI was associated with the colonization of a new population involving a strong bottleneck, then the newly founded population may have low genetic diversity from the outset. Although such a population might express high genetic load (Kirkpatrick and Jarne 2000), the low genetic diversity at viability loci could persist following an increase in population size in the absence of further migration, so that ID would remain low. This sort of

explanation has been suggested to explain the loss of SI in the North American populations of *Arabidopsis lyrata* in which ID may have been purged via a population bottleneck during the colonization of North America (Foxe et al. 2010). Similarly, in *Leavenworthia alabamica*, newly selfing populations showed reduced ID, probably as a consequence of long-distance colonization through seed dispersal (Busch 2005), an explanation confirmed by a later study revealing strong heterosis in the most geographically isolated and divergent selfing population (Busch 2006). As discussed below, however, our inferences concerning heterosis are not entirely consistent with this idea.

Second, it is possible that we have underestimated ID in the SC population of *L. cavanillesii*. We measured ID in relatively small families, and only for early-stage components for the life cycle of a relatively long-lived perennial species, in which ID might occur at later (unmeasured) stages (e.g. Lobo et al. 2015). Indeed, genetic load expressed in early life-history stages may be purged more easily than that in later life-history stages (Husband and Schemske 1996). We also measured traits on plants growing in the greenhouse, likely a benign environment in which ID may be less strongly expressed (reviewed in Armbruster and Reed 2005). Under field conditions, inbreeding depression might, for example, be enhanced by predation or parasite pressure sufficiently to disfavor a shift to complete selfing (Agrawal and Lively 2001; Campbell 2014; Carr and Eubanks 2014). Seed predation in natural populations of *L. cavanillesii* is severe, with up to 50% of its seeds lost to seed-predating weevils (M. Voillemot and J.R. Pannell, personal observations). If weevils preferentially eat the seeds of inbred individuals (e.g. Bello-Bedoy et al. 2011), ID in the field might actually be substantially higher than our estimate. Nevertheless, while we cannot directly rule out these possibilities, inbreeding depression at later life stages should restore the population towards Hardy-Weinberg equilibrium (Ritland 1990), yet adult plants of L. cavanillesii in the SC population continue to display high inbreeding coefficients in the field ($F_{IS}$ = 0.36; Voillemot and Pannell 2016). It thus seems unlikely that our estimates of low inbreeding depression are qualitatively inaccurate. Recall that ID was measured under the same conditions for the SI and SC

population, and that putative effects of a benign environment did not prevent the expression of ID by plants from the SI population.

Third, the transition to SC in *L. cavanillesii* may have been so recent that the SC population has simply not had time to respond to selection for an increased selfing rate. Under this scenario, mixed mating may reflect a transitional state toward increased selfing rates. Some aspects indeed point to a recent divergence between the SI and SC populations of *L. cavanillesii* (Chapter I). In particular, we did not observe any phenotypic changes in flower morphology towards a selfing syndrome, which one might have expected for a population that has lost SI (Chapter I). A similar scenario was suggested for *Leavenworthia alabamica* (Busch et al. 2011) and *Arabidopsis lyrata* (Hoebe 2009), both species in which a recent loss of SI was given as a possible explanation for the absence of a selfing syndrome in SC populations,. Similarly, in *Camissoniopsis cheiranthifolia*, the occurrence of outcrossing despite negligible ID in some populations might be due to the fact that they are still in the process of evolving higher levels of selfing (Dart and Eckert 2013).

*Variation in heterosis for self-compatible vs. incompatible populations*

Our study has found evidence for heterosis expressed in crosses between geographically relatively distant populations, for crosses involving both the SC and SI populations. Except for seed production, every trait measured in the SC population showed greater fitness when plants were crossed with those from a distant population. Crosses involving only SI populations also revealed heterosis, but somewhat less so overall, given that heterosis was not apparent for time to flowering or nectar quantity. These results are broadly consistent with expectations based on patterns of population differentiation (Figure 1b). In particular, while *L. cavanillesii* shows a clear signal of genetic isolation by distance for both SI and SC populations, population differentiation tends to be higher for pairs of populations involving the SC population (Figure 1b). Interestingly, we found no evidence for significant overall heterosis expressed in crosses between populations involving the SC population and sampled in close proximity (average cumulative index of

heterosis: 0.08). Here, only pollen production and plant growth showed evidence for any significant degree of heterosis. This result, too, is consistent with the pattern of isolation by distance found for *L. cavanillesii.* It suggests either that the shift from SI to SC has not erased this pattern, or that there has been continued migration among nearby populations that differ in their SI status. Again, these patterns recall those found by Busch (2006) for *Leavenworthia alabamica*, where strong reproductive isolation of one SC population resulted in high heterosis with other populations, whereas another more connected SC population did not. Taken together, our results suggest that the mating system alone is not sufficient to account for differences in heterosis between populations, but that population size or isolation are crucial parameters, too (Oakley and Winn 2012).

We found evidence for strong maternal effects on heterosis, with reciprocal crosses of SC x SI plants revealing contrasting results depending on which population contributed pollen and which contributed ovules to the cross. Indeed, when the SC population was the pollen donor, heterosis was negative, pointing to the expression of outbreeding depression. Outbreeding depression may be attributable to a breakdown in co-adapted gene complexes, perhaps due to local adaptation in this population (c.f. Escobar *et al.*, 2008; Walisch *et al.*, 2012). However, the fact that we observed outbreeding depression only when SC plants were the pollen donor points to asymmetrical selection on female vs. male functions. Evidence for a parental influence on offspring performance has been shown to influence individual genotypes in *Campanula rapunculoides,* where pollen production was mostly influenced by maternal effects (Good-Avila and Stephenson 2003). Reduced pollen production is expected to evolve after the transition to SC and a shift to higher rates of selfing, because of the reduced allocation of resources to pollen export (Charlesworth and Charlesworth 1981). Here, it is interesting to note that, in *L. cavanillesii,* flower and pollen production were influenced in opposite directions, depending on the direction of the cross. It would thus be interesting to conduct further reciprocal controlled crosses, not least to determine how generally our results apply to other populations of *L. cavanillesii* and, specifically, to assess whether asymmetrical selection is revealed only for the SC population, or for others too.

*Concluding remarks*

Theoretical and empirical work point to a change in ID as one of the main forces influencing the transition to selfing and the apparent stability of mixed mating (Charlesworth and Charlesworth 1987; Winn et al. 2011). Our observation of high ID for SI individuals *of L. cavanillesii* are consistent with the idea that selection against inbreeding in these populations might indeed prevent the spread of mutations conferring a capacity to self-fertilize in these populations. In contrast, our observations for the mixed mating population run counter to our expectations based on findings in other studies (Winn et al. 2011). It appears that mutations that might cause ID have largely been purged from the SC population, despite the fact that outcrossing rates continue to be high due to high pollinator visitation rates. It remains possible that L. cavanillesii continues to be selected for outcrossing and the maintenance of an outcrossing floral syndrome under variable pollinator availability, as has been suggested for other species (e.g. Kalisz and Vogler 2003, 2004; Dart et al. 2012). Nevertheless, the selection of expensive traits that maintain outcrossing is difficult to reconcile with the absence of ID observed in the SC population of *L. cavanillesii.* Ultimately, mixed mating in L. cavanillesii is more likely to be an outcome of a recent loss of SI, and the failure of natural selection, through lack of time and/or relevant genetic variation, to bring about a shift towards complete selfing and a selfing syndrome.

## ACKOWLEDGMENTS

We thank L. Liberati for help with phenotypic measurements, N. Ruch for help in the glasshouse, and Y. Cuenot for help with pollen counting. We are grateful to the University of Lausanne for funding the studentship awarded to MV.

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**Table 1. Summary of sampling design to estimate inbreeding depression in *Linaria cavanillesii*.** Number of families and number of plants assayed per family are shown for each treatment. Treatments were: self (self pollen), outcross-within (outcross pollen from a plant within population), outcross-between (pollen from another population, either DEN, COV, or BUI).

| | Treatment | Number of families | Number of plants assayed | | Treatment | Number of families | Number of plants assayed |
|---|---|---|---|---|---|---|---|
| **BUI (SI)** | Self | 3 | 11 | **COV (SC)** | Self | 10 | 42 |
| | Outcross between (COV) | 7 | 27 | | Outcross between (BUI) | 8 | 35 |
| | Outcross between (DEN) | 6 | 30 | | Outcross between (DEN) | 6 | 18 |
| | Outcross within | 6 | 30 | | Outcross within | 4 | 15 |
| Total | | | 98 | | | | 110 |

**Table 2. Summary of statistical results for all the fitness traits measured for 208 plants (nested within 52 maternal families), for one self-compatible (SC) and one self-incompatible (SI) population and resulting from hand self- and cross-fertilization**. Number of degrees of freedom (Df), F-ratios and p-values are reported. * indicates traits that have been analyzed using analysis of variances. Proportion of germination was analyzed using a generalized mixed model ($^{\#}$ represents $\chi^2$ instead of F-ratios); all other traits were analyzed using random linear models, with block and family treated as random factors. For growth, flower size and nectar measurement, group, temperature and hygrometry were added as random factors (see text for details). Significant results are highlighted in grey.

| | COV (SC) | | | BUI (SI) | | |
|---|---|---|---|---|---|---|
| **Traits** | **Df** | **F** | **p-value** | **Df** | **F** | **p-value** |
| **Seed number*** | 3,24 | 2.59 | 0.08 | 3,18 | 4.41 | 0.012 |
| **Seed weight*** | 3,23 | 1.57 | <0.22 | 3,18 | 1.08 | 0.38 |
| **Proportion germination$^{\#}$** | 3 | 39.42$^{\#}$ | <0.001 | 3 | 15.59$^{\#}$ | 0.002 |
| **Days to flowering** | 3, 76,99 | 0.30 | 0.83 | 3, 56,275 | 23.59 | < 0.001 |
| **Flower production** | 3, 80,20 | 1.14 | 0.34 | 3, 39.15 | 1.36 | 0.27 |
| **Growth after nine weeks** | 3, 81,45 | 6.43 | < 0.001 | 3, 81,23 | 5.95 | 0.001 |
| **Flower size** | 3, 121.95 | 4.75 | 0.004 | 3, 96.02 | 2.67 | 0.05 |
| **Nectar quantity** | 3,100.49 | 0.60 | 0.61 | 3,59.11 | 5.56 | 0.002 |
| **Nectar quality** | 3, 108.54 | 8.28 | < 0.001 | 3, 79.76 | 1.40 | 0.25 |
| **Flower longevity** | 3, 58.98 | 0.97 | 0.41 | 3, 45.91 | 3, 17 | 0.03 |
| **Pollen production** | 3, 76.9 | 7.81 | < 0.001 | 3, 23.0 | 1.45 | 0.25 |
| **Ovule production** | 3, 45.1 | 1.75 | 0.17 | 3,42.9 | 0.45 | 0.72 |
| **Pollen:ovule ratio** | 3, 23.8 | 5.70 | 0.004 | 3, 19.3 | 1.70 | 0.20 |

**Figure 1. a) Names and localities of the *Linaria cavanillesii* populations sampled in Spain** (SC: self-compatible population, SI: self-incompatible population, SI/leaky SI: leaky self-incompatible population). * indicates populations used for crosses to measure inbreeding depression and heterosis. **b) Pattern of isolation by distance revealed by analysis of microsatellite variation for populations of *L. cavanillesii* across its range, measured in terms of pairwise $F_{ST}$.** Each dot represents a pair of populations, with black dots representing distances between SC population and distant populations, and grey dots representing distances between SC population and nearby populations. Regression lines are shown for all pairs of populations (full line; Mantel test: p = 0.06) or pairs of SI populations only (dashed line; Mantel test: p = 0.002). Data plotted from Chapter I.

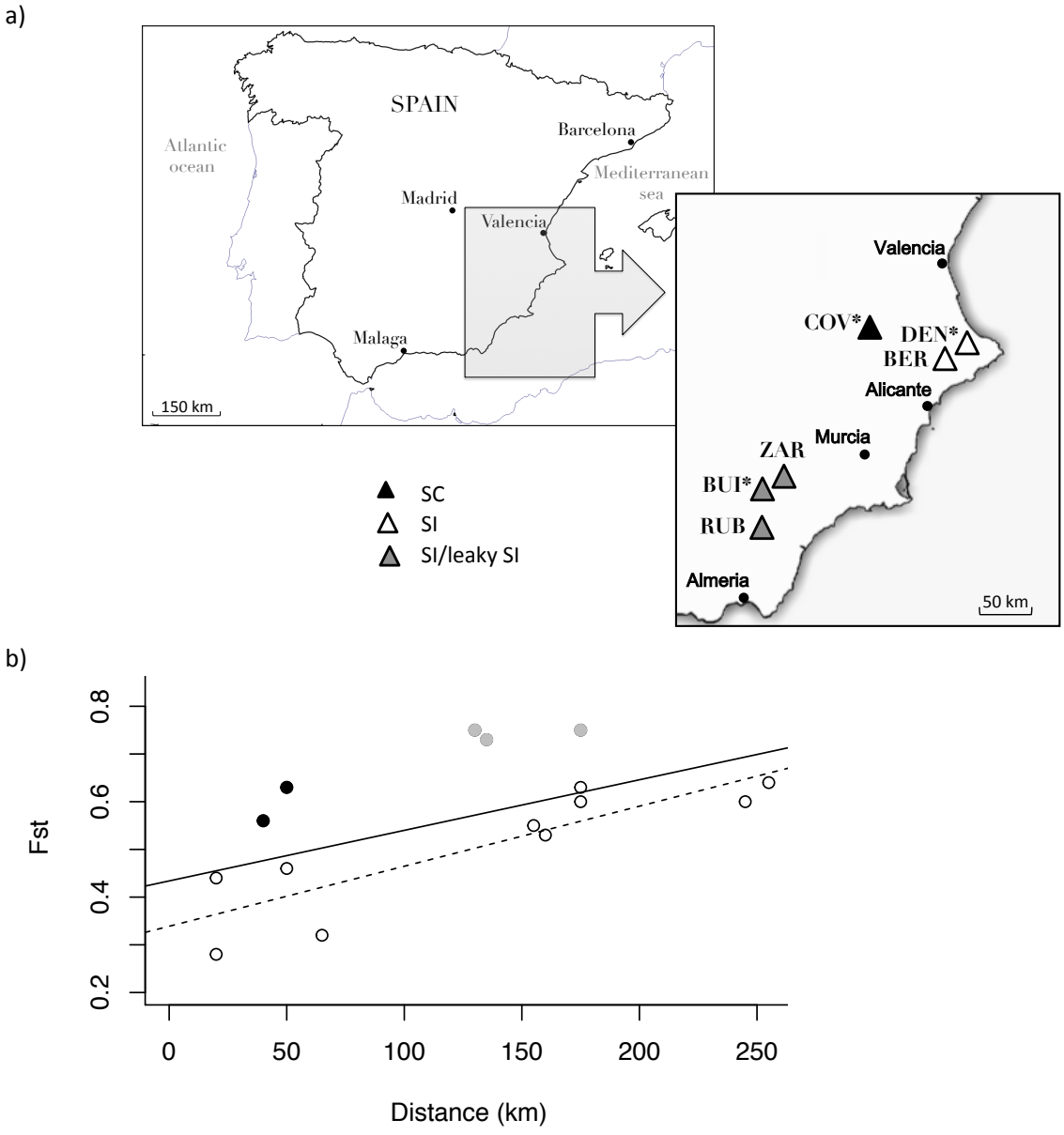


**Figure 2. Comparison of inbreeding depression for different phenotypic traits in one self-compatible (SC) population and one self-incompatible (SI) population of *Linaria cavanillesii*.** Values plotted were calculated as the means of per-family estimates of inbreeding depression. The cumulative fitness index combines the product of traits indicated with a #: proportion of plant germination, plant growth, number of flowers produced at the time of harvest and average of pollen and ovule production per flower. Stars indicate significant results after post-hoc tests ($P < 0.05$).

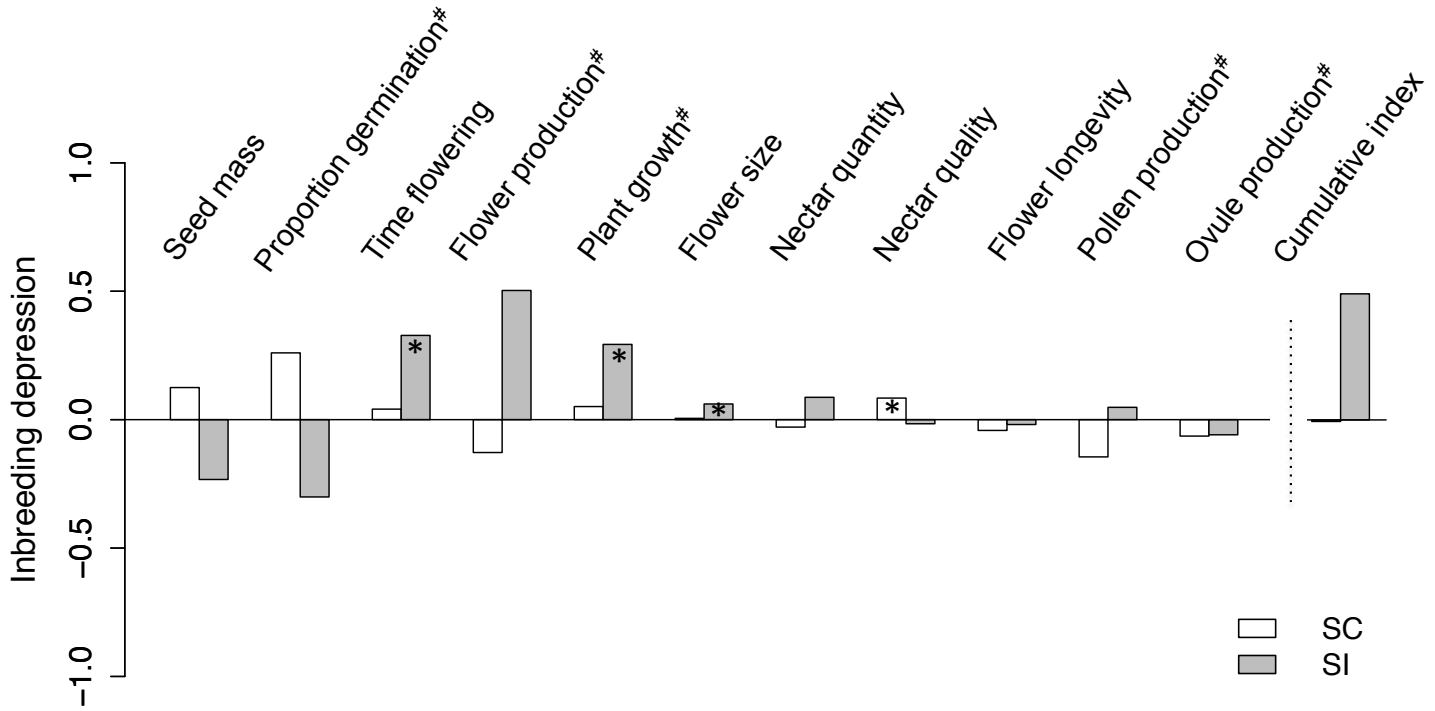
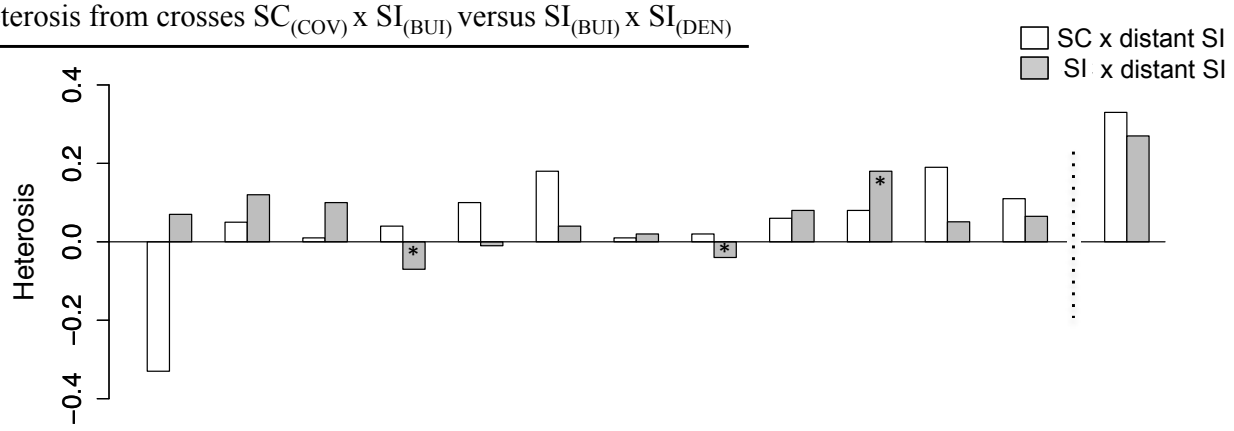
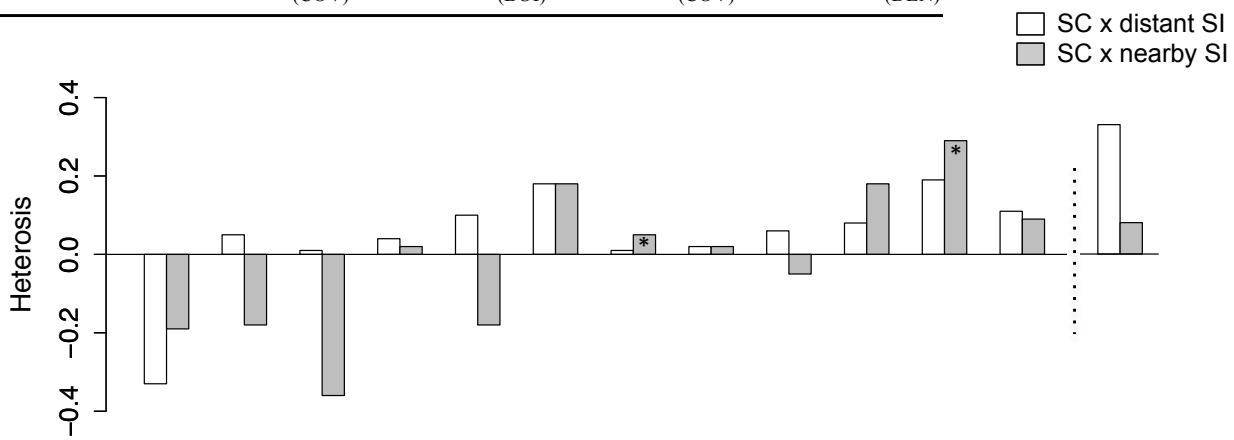


**Figure 3. Estimates of heterosis revealed by crosses between different populations of *Linaria cavanillesii*.** The first term describing the crosses always corresponds to the maternal plant and the second one to the pollen donor. (a) Heterosis revealed for crosses between geographically distant populations, where one population in the cross was SC (white; $DEN_{(SI)}$ - $COV_{(SC)}$) or both populations were SI (grey; $DEN_{(SI)}$ – $BUI_{(SI)}$). (b) Heterosis revealed for crosses involving the SC population with a distant SI population (white; $COV_{(SC)}$ - $BUI_{(SI)}$) or a nearby SI population (grey; $COV_{(SC)}$ – $DEN_{(SI)}$). (c) Heterosis revealed for respective crosses between the SC population and an SI population, with SC as the mother plant (white; $COV_{(SC)}$) or SI as the mother plant (grey; $BUI_{(SI)}$). Plotted values were calculated as the means of per-family estimates of heterosis. The cumulative fitness index combines the product of traits indicated with a $^{\#}$: seed production, proportion of seed germination, plant growth, number of flowers produced at the time of harvest and average of pollen and ovule production per flower. Stars indicate significant results after post-hoc tests ($P < 0.05$).

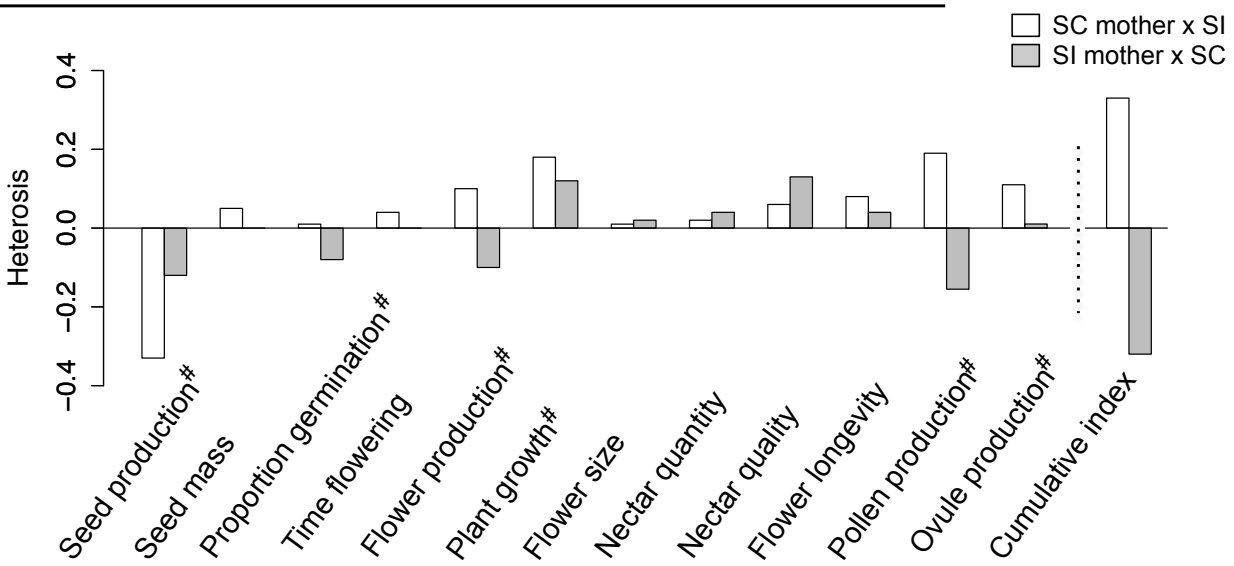
(a) Heterosis from crosses $SC_{(COV)}$ x $SI_{(BUI)}$ versus $SI_{(BUI)}$ x $SI_{(DEN)}$




(b) Heterosis from crosses $SC_{(COV)}$ x distant $SI_{(BUI)}$ versus $SC_{(COV)}$ x nearby $SI_{(DEN)}$




(c) Heterosis from crosses $SC_{(COV)}$ mother x $SI_{(BUI)}$ versus $SI_{(BUI)}$ mother x $SC_{(COV)}$



# SUPPLEMENTARY INFORMATION

**Figure S1. Comparison of (a) seed production and (b) seed mass, between two populations of *Linaria cavanillesii* following different crossing treatments (**SC: self-compatible; SI: self-incompatible; out_b: outcross between populations; out_w: outcross within population). Significant differences are indicated with no letters in common; bars indicate mean ± SEM. Number of replicates is indicated beneath each data point.

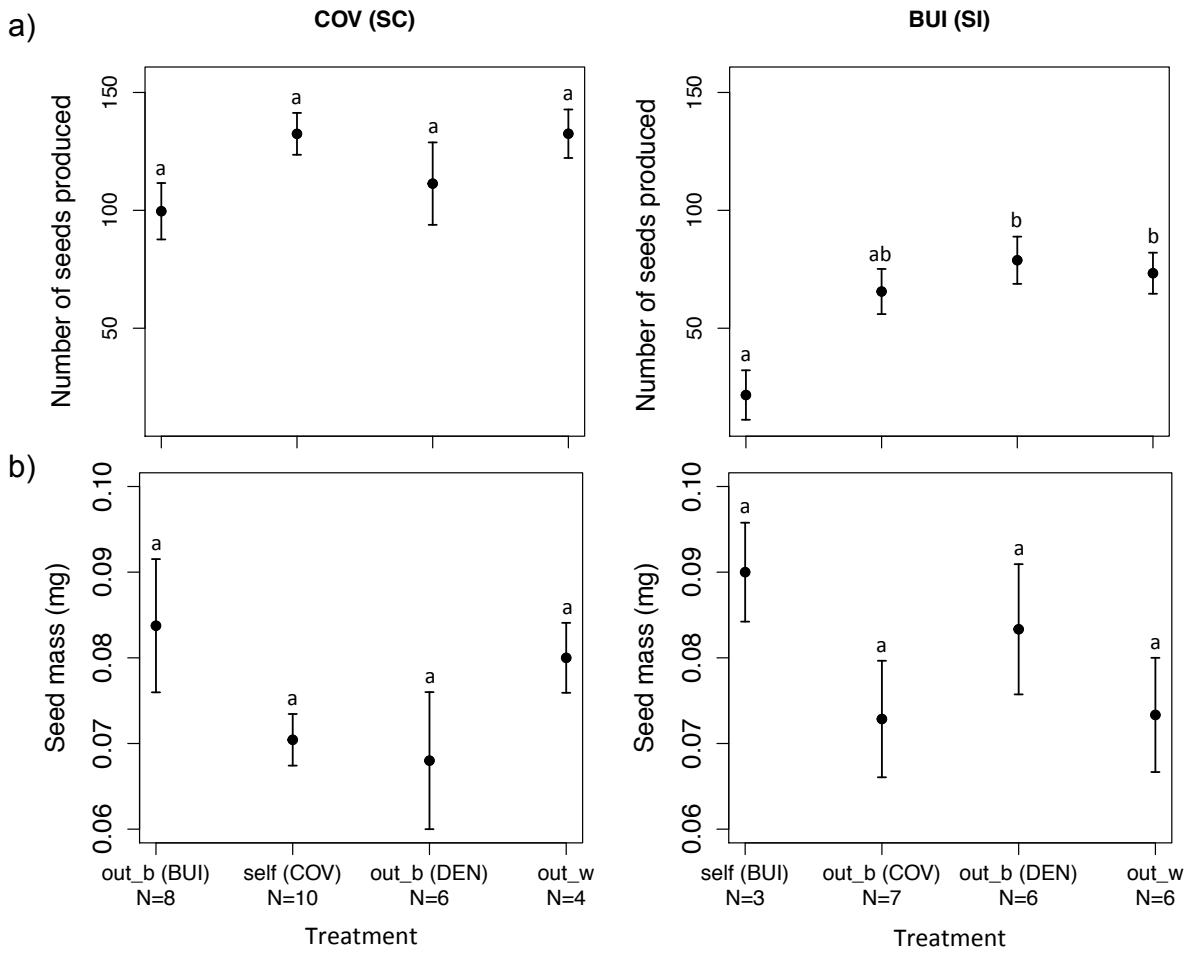


**Figure S2. Comparison of (a) number of days from germination to flowering and (b) total flower production overs three months, between two populations of *Linaria cavanillesii* following different crossing treatments (**SC: self-compatible; SI: self-incompatible; out_b: outcross between populations; out_w: outcross within population). Significant differences are indicated with no letters in common; bars indicate mean ± SEM. Number of replicates is indicated beneath each data point.

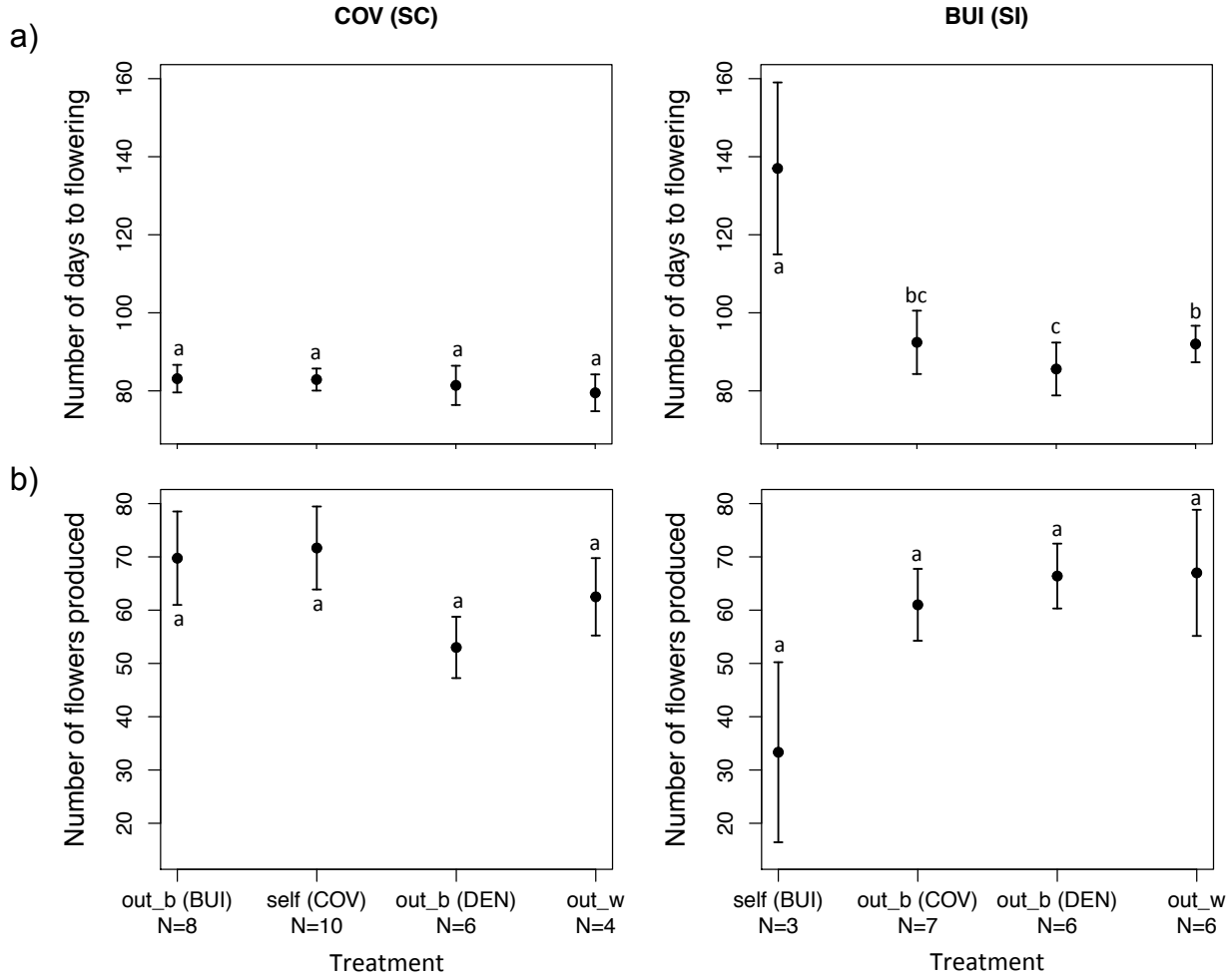


**Figure S3. Comparison of growth (difference with initial transplantation size) nine weeks after transplanting between two populations of *Linaria cavanillesii* following different crossing treatments** (SC: self-compatible; SI: self-incompatible; out_b: outcross between populations; out_w: outcross within population). Significant differences are indicated with no letters in common; bars indicate mean ± SEM. Number of replicates is indicated beneath each data point.

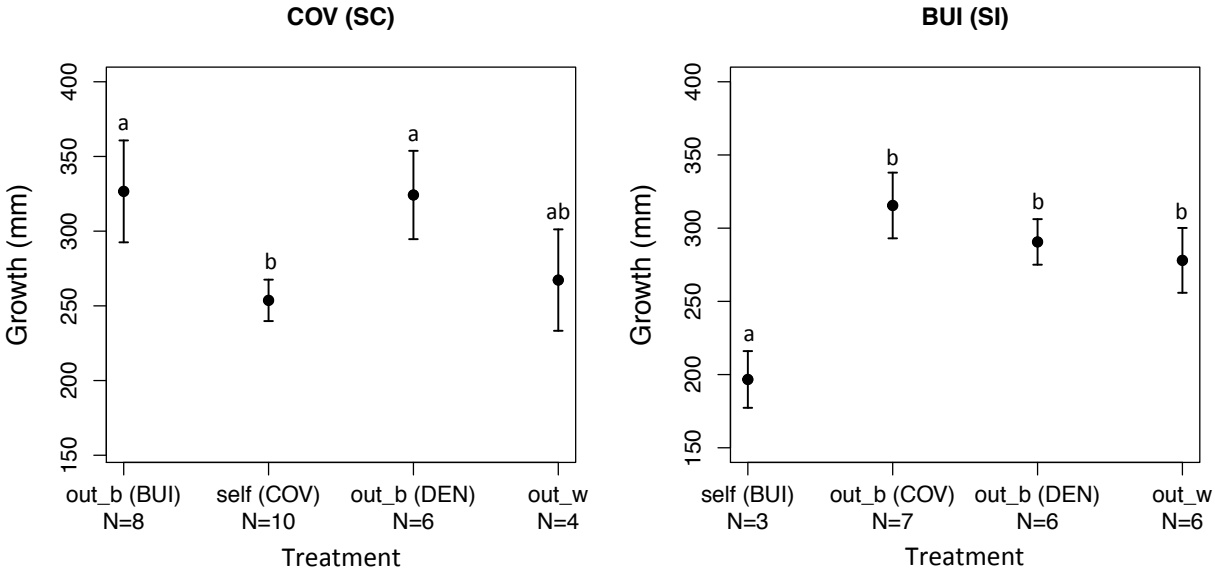


**Figure S4. Comparison of (a) flower size and (b) flower longevity, between two populations of *Linaria cavanillesii* following different crossing treatments** (SC: self-compatible; SI: self-incompatible; out_b: outcross between populations; out_w: outcross within population). Significant differences are indicated with no letters in common; bars indicate mean ± SEM. Number of replicates is indicated beneath each data point.

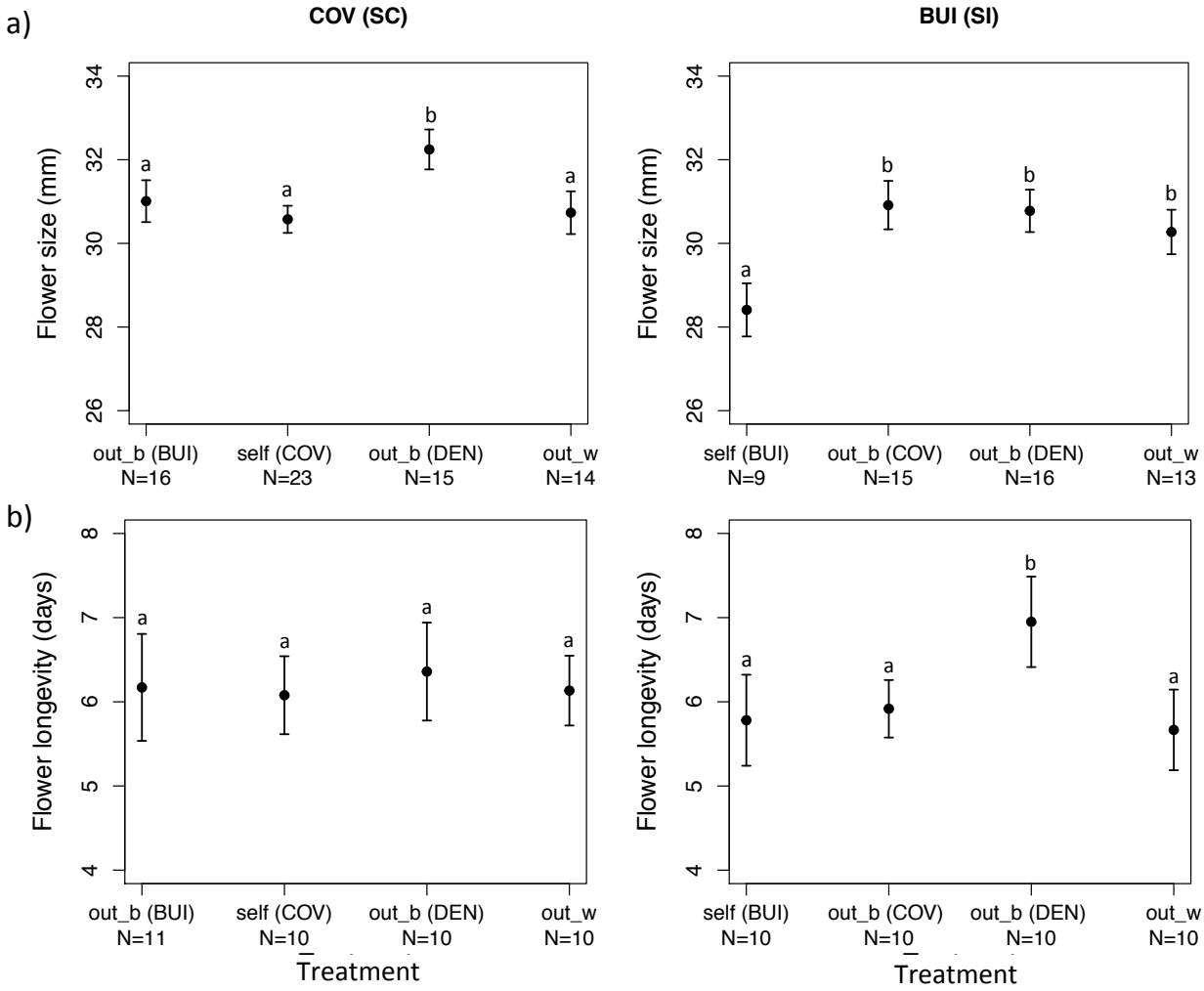


**Figure S5. Comparison of (a) nectar quantity and (b) nectar quality, between two populations of *Linaria cavanillesii* following different crossing treatments** (SC: self-compatible; SI: self-incompatible; out_b: outcross between populations; out_w: outcross within population). Nectar quantity was measured as the length of spur showing nectar. Significant differences are indicated with no letters in common; bars indicate mean ± SEM. Number of replicates is indicated beneath each data point.

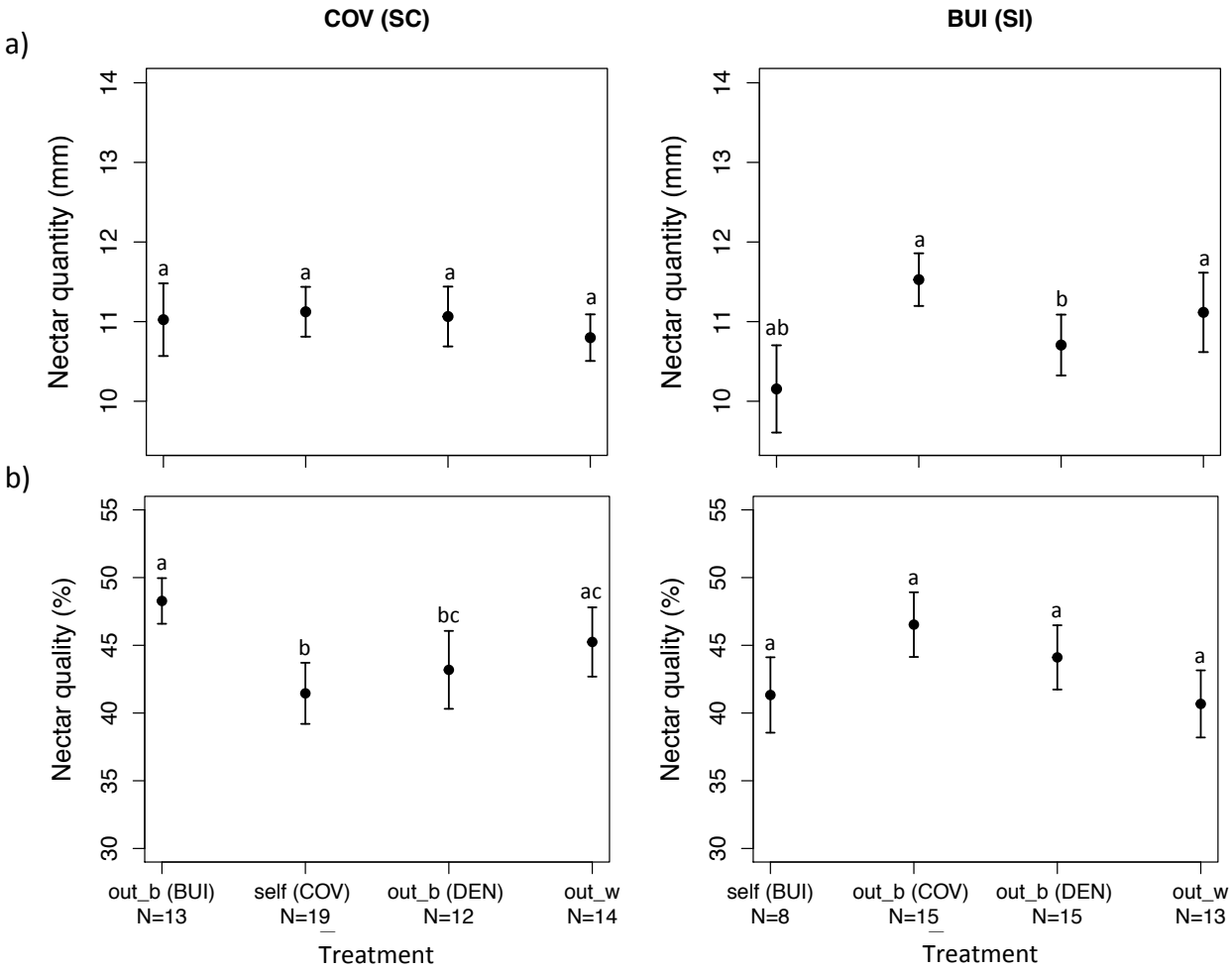


**Figure S6. Comparison of (a) ovule number and (b) pollen grains production, between two populations of *Linaria cavanillesii* following different crossing treatments** (SC: self-compatible; SI: self-incompatible; out_b: outcross between populations; out_w: outcross within population). Significant differences are indicated with no letters in common; bars indicate mean ± SEM. Number of replicates is indicated beneath each data point.

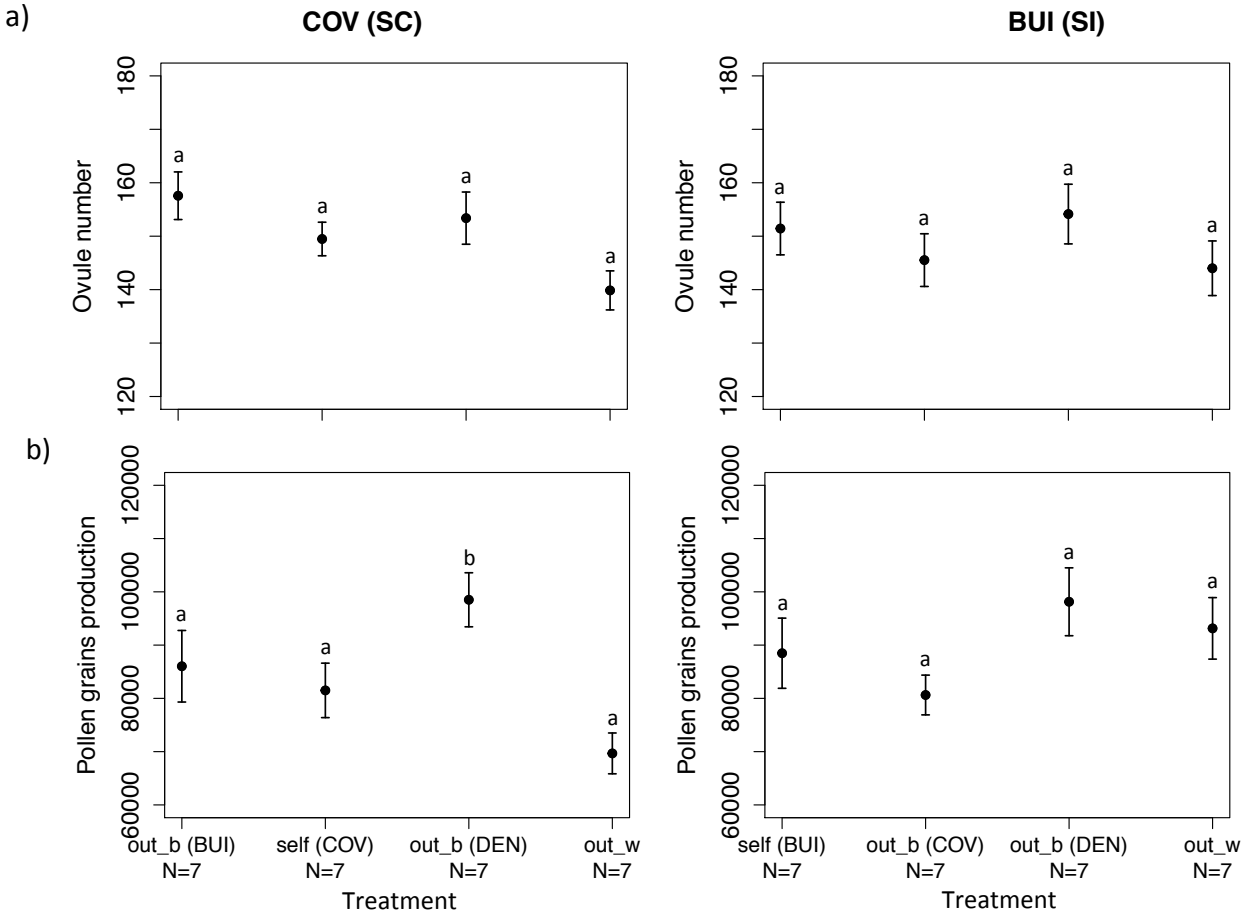
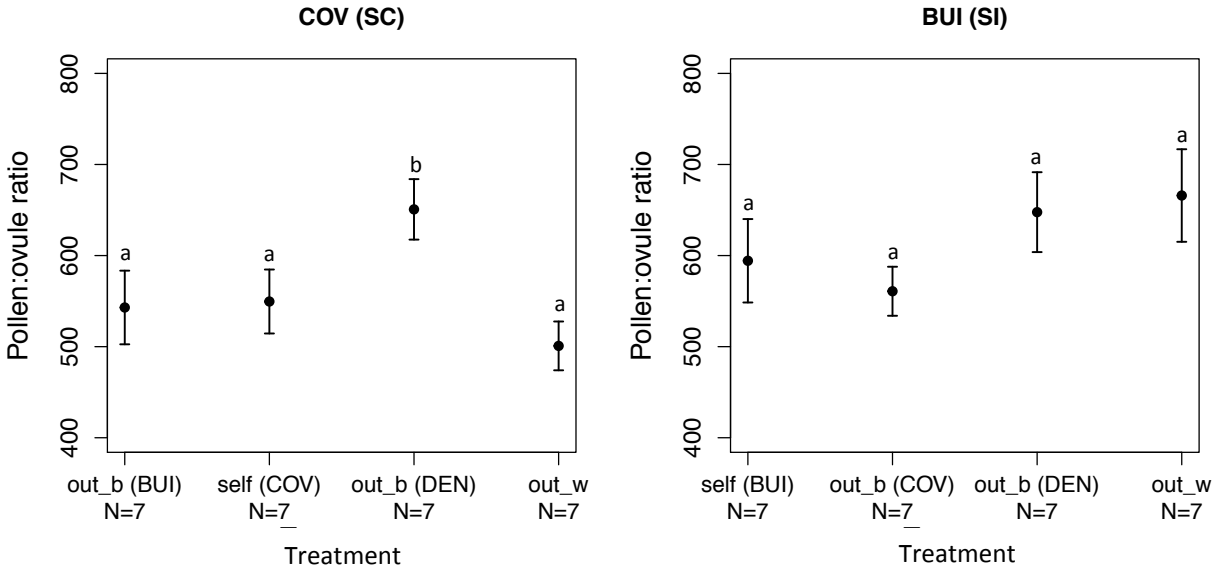


**Figure S7. Comparison of pollen:ovule ratio between two populations of *Linaria cavanillesii* following different crossing treatments** (SC: self-compatible; SI: self-incompatible; out_b: outcross between populations; out_w: outcross within population). Significant differences are indicated with no letters in common; bars indicate mean ± SEM. Number of replicates is indicated beneath each data point



# - CHAPTER III -

## The divergence history of the perennial plant *Linaria cavanillesii* confirms a recent loss of self-incompatibility

M. Voillemot, C. Roux, Q. Rougemont and J. R. Pannell

*(In preparation)*

# ABSTRACT

Many plants prevent inbreeding by way of a self-incompatibility (SI) system, but the loss of SI, and thus reversions to self-compatibility (SC), have been very frequent in the evolutionary history of angiosperms. The loss of SI will often provoke an increase in the selfing rate, and is often followed by purging of inbreeding depression and the gradual evolution of a selfing syndrome, where plants have smaller flowers with reduced pollen and nectar production. In this study, we used approximate Bayesian computation (ABC) to estimate the timing of divergence between populations of the long-lived perennial plant *Linaria cavanillesii,* a species with populations that differ in their SI status and in which the loss of SI has coincided with the purging of inbreeding depression but not with a transition to full selfing or with the evolution of a selfing syndrome. Our analysis indicates that divergence of the mixed-mating SC population from SI populations was indeed very recent, probably a result of a colonization bottleneck that facilitated the purging of deleterious mutations, but that there has likely been insufficient time for the evolution of increased selfing rates and a selfing syndrome.

**Keywords**: ABC simulations, mating-system transition, population divergence, purging, selfing syndrome, self-compatibility

# INTRODUCTION

Hermaphrodite plants have evolved numerous strategies to prevent self-fertilization and thus to avoid the deleterious effects of inbreeding depression (Barrett 2002). Perhaps the most effective of these is self-incompatibility (SI), which involves the rejection by pistils of pollen produced by the same genotype (Franklin-Tong 2008; Shimizu and Tsuchimatsu 2015). SI is widespread in flowering plants (60% of angiosperm species are considered SI, Igic and Kohn 2006), but it has also broken down numerous times. Indeed, the loss of SI probably represents the most common evolutionary transition to have occurred in the history of the angiosperms (Stebbins 1974; Igic et al. 2008; Goldberg et al. 2010; Wright and Barrett 2010).

Two main reasons have been given to explain the frequent transition from SI to SC observed in plants. The first is the direct fitness advantage gained by SC individuals that pass an extra copy of their genes to the next generation in their self-fertilized seeds (automatic selection hypothesis; Fisher 1941). Mutations conferring SC should thus spread in a population in the absence of countervailing forces, such as inbreeding depression (Charlesworth and Charlesworth 1987) or pollen discounting (Nagylaki 1976; Holsinger 1988; Harder and Wilson 1998; Porcher and Lande 2005), whereby self-fertilization compromises a plant's outcrossing potential (Harder and Barrett 1995). Although the advantage of automatic transmission provides a compelling reason to expect frequent shifts from SI to SC (Stone et al. 2014), outcrossing populations tend to express high inbreeding depression upon inbreeding (Husband and Schemske 1996; Winn et al. 2011), and it seems clear that for a mating-system transition to be selected, inbreeding depression must first be purged (see below; Lande and Schemske 1985; Crnokrak and Barrett 2002; Benesh et al. 2014).

The second hypothesis for the loss of SI, the reproductive assurance hypothesis (Darwin 1876; Lloyd 1965; Jain 1976; reviewed in Busch and Delph 2012), is linked to mating opportunities. In SI individuals,

the number of available partners depends both on population size as well as the presence of external vectors to move pollen from an individual to the other. In small and isolated populations, SI alleles can be lost by drift (Wright 1939), so that different individuals are more likely to carry the same alleles and therefore reject each other's outcross pollen, with consequently lost mating opportunities (Charlesworth and Charlesworth 1979; Busch and Schoen 2008; Young and Pickup 2010; Stone et al. 2014). Mates may also be limited during episodes of colonization, where single individuals establish in new localities, such as on islands (Baker 1955; Pannell 2015; Pannell et al. 2015). Similarly, in environments where pollinators are scarce, outcrossing may also become difficult (Baker 1955; Cheptou and Massol 2009; Massol and Cheptou 2011). In such situations, SC individuals may have a fitness advantage over SI individuals, particularly if they are able to self-fertilize autonomously, even if selfing leads to inbreeding depression (e.g. Herlihy and Eckert 2002 but see Layman et al. 2017). Understanding why some populations are resistant to the establishment of SC individuals, whereas SC fixes in others, is a central question in our quest to understand the diversity of plant mating systems.

Major changes are expected to follow a transition from SI to SC and a concomitant increase in the selfing rate. Because plants that are able to self-fertilize do not need pollinators, the evolution of SC and self-fertilization is typically associated with the evolution of a reduced investment in pollinator attraction and reward, such as through smaller flowers, lower pollen:ovule ratios and reduced nectar production (Ornduff 1969; Goodwillie et al. 2010; Sicard and Lenhard 2011). This 'selfing syndrome' has been reported in numerous plant species (reviewed in Goodwillie et al. 2010), and appears to be able to evolve rapidly after a transition to self-fertilization (Bodbyl Roels and Kelly 2011). However, not all species seem to experience a rapid shift in flower morphology towards the selfing syndrome (e.g. Busch 2005; Fenster et al. 2007; Dart et al. 2012), and the question of why some species do and others do not remains puzzling.

Following a transition to SC, deleterious mutations are expected to be quickly purged by selection, thus resulting in decreased inbreeding depression (Lande and Schemske 1985; Barrett and Charlesworth 1991). This is because selfing leads to increased homozygosity, with the expression of, and selection against, deleterious recessive alleles that would otherwise largely be masked from selection in heterozygous individuals. Inbreeding depression has indeed been found to be lower in predominantly selfing than outcrossing species (reviewed in Husband and Schemske 1996). Although purging can occur quickly, especially if the transition to selfing coincides with strong bottlenecks (e.g. Busch 2005; Guo et al. 2009; Ness et al. 2010), many partially selfing species maintain high levels of inbreeding depression for long periods of evolutionary time (Winn et al. 2011). This might be because inbreeding depression in such populations is so strong that no selfed individuals survive to reproductive maturity, so that there is thus no possibility for selection among selfed progeny that differ in their load of deleterious mutations (Lande et al. 1994). Alternatively, inbreeding depression might be purged over a longer period of time (Busch 2005b).

The consequences and timing of a transition toward SC have been investigated in a handful of pairs of plant species (e.g. Tang et al. 2007; Foxe et al. 2009; Guo et al. 2009). For instance, *Capsella grandiflora* (Foxe et al. 2009; Guo et al. 2009), *Clarkia xantiana spp. parviflora* (Runions and Geber 2000) and *Arabidopsis thaliana* (Tang et al. 2007) are SC species that have been recently derived from their SI related species, and all show evidence of a selfing syndrome across their geographic range (Runions and Geber 2000; Foxe et al. 2009). Moreover, studies of several species that show within-species variation in their SI status have also been revealing (e.g. Foxe et al. 2010; Ness et al. 2010; Busch et al. 2011). For instance, coalescent-based analyses revealed that *Leavenworthia alabamica,* a predominantly SI species, experienced a transition to SC in two populations as recently as over the last 150,000 years (Busch et al. 2011). Interestingly, the most recent population of *L. alabamica* to undergo the transition to SC (about 48,000 years ago) also shows negligible changes in floral morphology. Similarly, using nuclear genes, chloroplast markers and nuclear microsatellites, Foxe et al. (2010) found that some populations of

*Arabidopsis lyrata* probably evolved higher selfing rates over the last 10, 000 years, also without showing any accompanying shift towards a selfing syndrome.

The perennial herb *Linaria cavanillesii* shows variable levels of SI among its populations and underwent a recent breakdown of the SI system in part of its range (Chapter I). The one known SC population of *L. cavanillesii* appears to be at an intermediate step of its evolution from SI to self-fertilization. On one hand, a comparison between SI and SC populations found that the SI population is accompanied by high inbreeding depression, whereas crosses between SC individuals reveal no evidence of inbreeding depression, suggesting that the SC population has been largely purged of deleterious mutations (Chapter II). On the other hand, the SC population continues to show substantial levels of outcrossing (selfing rate s = 0.59; Chapter I), a situation in which the purging of inbreeding depression might be ineffectual (Winn et al. 2011). Nor does the selfing population show any evidence of a selfing syndrome, with flowers from both SC and SI populations producing large flowers with a long nectar-containing spurs, and no reduction in pollen production (Chapter I). Together, these observations suggest that the shift to SC is sufficiently old to have purged mildly deleterious mutations in SC populations, but not recent enough for the evolution of a selfing syndrome of reproductive traits.

To shed the light on the dynamic of transition from SI-associated traits to SC, we estimated the timing of the transition from SI to SC in *L. cavanillesii*. Specifically, we used an approximate Bayesian computation framework (ABC) (Beaumont et al. 2002) to infer the time of split between SI and SC populations, on the basis of molecular variation at 16 microsatellite loci. ABC uses coalescent simulations to generate genetic data under a given demographic model. Simulated data are then compared with observed data to test the relevance of the demographic model, bypassing the need to compute full likelihoods – a major limitation for most demographic models (Beaumont et al. 2002; Csilléry et al. 2010). Our results suggest that the SC population is recently derived from neighboring SI populations,

and contribute to an understanding of the mating-system transition likely to have taken place in *L. cavanillesii.*

# MATERIAL AND METHODS

*Study species*

*Linaria cavanillesii* is a perennial herb endemic to southeastern Spain. Populations are strongly isolated (Chapter I), and grow on north-northwest-oriented cliffs at sites ranging from 300 to 1400 m in altitude. Because populations are sparse and small, the species has a protected conservation status (Sánchez-Gómez et al. 2003). Between May and June, individuals grow multiple herbaceous shoots from a sub-woody perennial base, with inflorescences developing towards the end of the branches. Yellow flowers have long floral nectar spurs and are pollinated by a variety of bees and bumblebees. Around 30 days after fertilization, mature capsules open and seeds are dispersed passively, probably aided by wind.

*Population sampling*

Six populations of *L. cavanillesii* have been the focus of detailed previous analysis (Chapter I). These include a fully SC population (COV), two SI populations (BER, DEN), and three mixed populations with both SI and leaky SI plants (BUI, RUB, ZAR) (Chapter I). The species has a strong population genetic structure, with mean pairwise $F_{ST}$ = 0.56 (min $F_{ST}$ = 0.32, max $F_{ST}$ = 0.75), and minimum $F_{ST}$ for the SC populations with each of the two closest and northernmost SI populations ($F_{ST}$ = 0.63 and 0.56 for COV-DEN and COV-BER, respectively; Chapter I). We focused the current study on these three northern populations (i.e., COV, BER, DEN; Figure 1).

We collected leaf material from 28 to 41 individuals from each of the three natural populations, and extracted DNA with the DNeasy 96 Plant kit (Qiagen). Sixteen polymorphic microsatellite markers were

amplified by PCR (Biometra thermocycler) using the following reagents: 1 x PCR mix: 2 ng/µL template DNA, 10 x PCR Buffer, 25 mM $MgCl_2$, 5 x Q-solution, 2.5 mM dNTP, 0.2 µM of each primer and 0.5 U/µL of Taq DNA polymerase (HotStarTaq®, Qiagen). The thermocycling conditions were set to 15 min at 95° followed by 32 cycles of 30 sec at the annealing temperature (Chapter I), 30 sec at 72 °C and 30 sec at 95 °C, followed by one cycle of 1 min at the annealing temperature and a final extension of 30 min at 72 °C. PCR products were then sequenced on an ABI3100 sequencer (Applied Biosystems). We used the program Genemapper® to analyze microsatellite data.

*ABC procedure*

Two model comparisons were performed by using ABC to (1) test the possibility of gene flow among populations and (2) test alternative topologies of population trees. For each test, a specific ABC approach was adopted.

*ABC test of gene flow between natural populations*

We tested the hypothesis of gene flow between *L. cavanillesii* populations by estimating the relative posterior probabilities of two models: model I (isolation), and model IM (isolation/migration). Model I pertains to an ancestral panmictic population that becomes subdivided in two isolated daughter populations with no gene flow between them (Figure 2). In model IM, by contrast, the two daughter populations remain connected by gene flow after an ancestral split (Figure 2). Specifically, under the model IM, gene flow occurs in both directions at independent rates $M_i = 4N_im$, where $M_i$ is the number of migrants received each generation by population *i,* $N_i$ is the effective population size of population *i*, and *m* is the proportion of individuals in population that were migrants from the other population the previous generation. The effective population size of the two current populations and the ancestral one were randomly sampled from the uniform prior [0 – 3,000] individuals. The time of split was sampled from the

uniform prior [0 – 100,000] generations. The number of migrants under the IM model rate was sampled from the uniform prior [0 – 20].

For each demographic model, we used the software ms to perform one million multi-locus coalescent simulations (Hudson 2002). The program's output was then converted in microsatellite datasets on the basis of a generalized stepwise mutation model (GSM) assuming a mean mutation rate $\mu = 2.5 \times 10^{-4}$ (based on current knowledge about estimates of μ in plants; e.g. Vigouroux et al. 2002; Marriage et al. 2009). The probability of an increase or decrease in the repeat number for each mutation was modeled assuming a geometrical parameter α distributed following a uniform prior and sampled on the interval [0 – 0.5]. For each simulated and observed dataset, an array of summary statistics for polymorphism and divergence data were computed using publicly available R-scripts (Illera et al. 2014; Rougemont et al. 2016; https://github.com/QuentinRougemont/MicrosatDemogInference). The computed summary statistics correspond to the mean and standard deviation of the number of alleles over the sixteen loci (*A*), the allelic richness ($A_r$), the observed and expected heterozygosities ($H_o$ and $H_e$), the allele size in base pairs, the Garza-Williamson index (GW*;* Garza and Williamson 2001), $G_{ST}$ (Nei 1973), and $\delta\mu_2$ (Goldstein et al. 1995).

Relative posterior probabilities of models I and IM were estimated from comparisons between the vector of observed summary statistics and a reference table comprising summary statistics computed from two million simulations (one million simulations under each model). The 500 replicate simulations (out of 2 x 1,000,000) falling nearest to the observed summary statistic values were retained, and these were weighted by an Epanechnikov kernel that peaks when $S_{observed} = S_{simulated}$. From the retained simulations, posterior probabilities of each model were estimated using a feed-forward neural network implementing a nonlinear multivariate regression by considering the model itself as an additional parameter to be inferred under the ABC framework, using the R package "abc" (Csilléry et al. 2012). Computations were performed using 50 trained neural networks and 15 hidden networks in the regression. The robustness of

the ABC model comparison (i.e., the probability of correctly supporting a model M given an estimated posterior probability *P*) was evaluated as follows: 1,000 pseudo-observed datasets (PODs) were randomly simulated under each compared model using similar prior distributions. To estimate the relative posterior probabilities of each model, each simulated POD was then treated by ABC in a way similar to that of the *Linaria* dataset. Finally, we estimated the robustness, *R*, of our inference on the basis of the obtained empirical distribution of the posterior probability *P* under the two models, with $R = P(P_I = P \mid I) / [ P(P_I = P \mid I) + P(P_I = P \mid IM)]$. $P(P_I = P \mid I)$ is the probability of correct support for model I with the observed posterior probability $P_I$, and $P(P_I = P \mid IM)$ is the probability or wrongly supporting model I with the observed posterior probability *P*, assuming here that model IM is the correct model (Fagundes et al. 2007).

*DYABC test of alternative topologies in population trees*

The previously described approach is robust to test the existence of migration between two sampled gene pools, a feature not implemented to date in the software DYABC (Cornuet et al. 2014). However, preliminary analysis has shown that DYABC has a greater power to correctly estimate parameters of a model with no gene flow and is able to deal with a number of populations > 2 as in the *Linaria* system surveyed here. Thus, after rejecting model IM (i.e., the hypothesis of ongoing gene flow; see the Results section), we tested alternative topologies describing possible histories of BER, COV and DEN populations (Figure 3a). This was achieved using the software DYABC (v.2.0.4) (Cornuet et al. 2014). We compared three alternative topologies of a model of the evolution of three populations (Figure 3a). This model describes the subdivision $T_2$ generations ago of an ancestral population of size $N_5$ into two populations, Pop-3 and Pop-4. Pop-3 has a constant effective population size $N_3$ from the time of the ancestral split up to the present. In contrast, Pop-4 of size $N_4$ is split into the two daughter populations Pop-1 (of size $N_1$) and Pop-2 (of size $N_2$) $T_1$ generations ago. All three possible topologies of this model were compared, with Pop-3 being either BER, COV or DEN (Figure 3a). All topologies shared the same

prior distributions and were randomly simulated 300,000 times, with parameters values sampled from uniform distributions. $N_1$, $N_2$, $N_3$, $N_4$ and $N_5$ were sampled from the uniform prior [0 - 30,000] individuals. The oldest time of divergence, $T_2$, was sampled from the uniform prior [0 - 50,000] generations. Finally, $T_1$, the most recent time of divergence was sampled for each iteration *i* from the uniform prior [0 - $T_{2\text{-i}}$], where $T_{2\text{-i}}$ is the sampled value for $T_2$ at the iteration *i*. A generalized stepwise mutation model (Estoup et al. 2002) was adopted with a mutation rate of $\mu = 1.25 \times 10^{-5}$. Finally, an array of summary statistics were computed for each iteration obtained for all topologies. These statistics were: the mean number of alleles; the genetic diversity (Nei 1973); the mean allele size variance; the Garza-Williamson index (GW; Garza and Williamson 2001); $F_{ST}$ (Weir and Clark Cockerham 1984); the mean classification index (Pascual et al. 2007); the shared allele distance between populations (Chakraborty and Jin 1993) and the $\delta\mu^2$ distance (Goldstein et al. 1995).

The relative posterior probabilities of the three topologies were estimated using the logistic regression estimate implemented in DIYABC (Fagundes et al. 2007; Beaumont 2008) from the 1,000 simulated datasets closest to the observation. We used a goodness-of-fit test to evaluate the capacity of the estimated parameters to reproduce the observed dataset under the best-supported scenario. 1,000 datasets were simulated by taking a random combination of parameters from the estimated joint posterior distribution. Summary statistics were then computed for each of the 1,000 simulated datasets in order to empirically obtain the expected distribution of the chosen statistics under the estimated scenario.

# RESULTS

Our ABC framework provides a strong statistical rejection of a hypothesised scenario involving ongoing gene flow between the SC population COV and the two geographically nearby SI populations DEN ($P_{Isolation}$ = 0.938; robustness = 1; Table 1) and BER ($P_{Isolation}$ = 0.657; robustness = 1; Table 1). Our analysis also provides strong statistical support for a scenario of ongoing gene flow between the two SI

populations BER and DEN ($P_{Migration}$ = 0.91; robustness = 1; Table 1). Here, robustness represents the probability of correctly supporting a model M given the posterior probability $P_M$, estimated by ABC, and can be interpreted as being 1 minus the *P*-value.

Using the software DIYABC (Cornuet et al. 2014), we compared different topologies of a three-population model of divergence (Figure 3a). Two successive population splits occur under this model, at two different times in the past $T_1$ and $T_2$, leading to three current populations from a single ancestral one. The best-supported topology on the basis of our ABC analysis corresponds to an ancestral split into two SI populations followed by a more recent split between the current SC population COV and current SI population BER. This scenario is supported with a relative posterior probability of 0.69 [0.66-0.73]. The alternative scenarios 1 and 2 (Figure 3a) are supported with posterior probabilities of 0.24 [0.20-0.27] and 0.07 [0.06-0.09], respectively.

Finally, we estimated parameters of the best-fitting topology using the same statistical framework implemented in DIYABC (Figure 3b). Assuming a mutation rate of 1.25 x $10^{-5}$, population sizes of current populations were estimated to be $N_{BER}$ = 3,460 (95% CI: 1,120 - 11,700), $N_{COV}$ = 850 (95% CI: 193 - 2,580) and $N_{DEN}$ = 9,640 (95% CI: 4,750 - 19,800) individuals (Figure 3b). The age of the ancestral split leading to the two current SI lineages was estimated to occur 10,800 generations ago (95% CI: 4,060 - 31,300; Figures 3b and S1). The split leading to the current SC COV population was estimated as having occurred 2,810 generations ago (95% CI: 714 - 14,400; Figures 3b and S1). Goodness-of-fit test represents the probability of correctly supporting a model given the estimated parameters, and the test was here successfully reproducing the observed dataset under the best-supported scenario (Figure S2 and S3). All of the 36 observed values for the chosen summary statistics could be reproduced under the estimated parameters for the best-supported scenario (Table S1). This result suggests that the proposed scenario was able to faithfully explain most of the patterns of polymorphism and divergence observed within natural

population of *L. cavanillesii*, even though it lacks the complexity of the populations' real demographic history.

# DISCUSSION

In this study, we used approximate Bayesian computation (ABC) simulations to compare the fit of alternative demographic models of divergence to empirical data of the plant *Linaria cavanillesii*, a species with among-population variation in its SI status (Chapter I). Overall, our analyses are consistent with a scenario where the lineage ancestral to COV (mixed mating SC) has been diverging from the SI populations for an estimated 2,810 generations. Our analyses reject a scenario of ongoing gene flow between the SC and each of the two SI populations, but are consistent with a scenario of ongoing gene flow between the two SI populations. Our simulations also suggest that the effective population size of SC population is about 10 times smaller than the SI populations and the inferred ancestral population. The inferred divergence time between DEN (SI) and the pair of populations COV (SC) and BER (SI) was nearly four times greater than between COV (SC) and BER (SI).

*Accuracy and robustness of our results*

Given that they are based on simple models of divergence and assumptions that likely diverge from reality, we can ask how reliable our inferences are. A critical step in ABC inference is to test whether the competing models can be accurately distinguished from one another. Our estimates of robustness based on the traditional ABC approach clearly indicate that the model choice procedure was reliable. Despite the simplifications we have made in constructing our models, the best-supported scenario was able to faithfully reproduce all of the 36 statistics available to describe patterns of polymorphism and divergence in natural populations of *L. cavanillesii*. This gives us some confidence that most of the historical signal is captured by the assumed modelling scenarios. Specifically, the absence of migration between the SC and

SI populations points to strong isolation and allows an estimate of the divergence time between the two populations.

An important result of our analysis is the inference that divergence between the SC and the SI populations began relatively recently. This estimate is of course sensitive to assumptions made for the mutation rate at markers chosen for the analysis. We assumed a mutation rate for our microsatellites ($1.25 \times 10^{-5}$) that corresponds to a lower bound of values estimated for other plant species (e.g. Vigouroux et al. 2002; Marriage et al. 2009). Lower mutation rates would elevate the estimated divergence time proportionally, but it would seem more likely that the mutation rate is in fact higher than the one assumed for *L. cavanillesii*, and thus that divergence times between SC and SI populations may be even lower than our estimate. Clearly, divergence-time estimates based on other markers would be valuable, but we do not expect that our inference of recent divergence of SC from SI populations would be radically altered.

Our analysis made evidently robust estimates for other model parameters, including the effective population sizes of the populations concerned and migration rates. Here, it is important to emphasize that these estimates are based on the assumption of uniformity in the population sizes and rates modeled. However, migration and mutation rates are known to vary substantially across the genome (Hodgkinson and Eyre-Walker 2011), and such variation has only recently been included in demographic inference approaches (Cruickshank and Hahn 2014; Roux et al. 2016). Moreover, mutation rates, effective population sizes and migration rates in natural populations are also likely to be heterogeneous over time, and it is not clear how such variation should impact on the details of our results. Nevertheless, our inferred parameter values conform to patterns we might expect, both in light of theory (Nordborg and Donnelly 1997; Charlesworth and Pannell 2001) and conclusions reached from other studies (Glémin et al. 2006; Wright et al. 2008; Duminil et al. 2009). In particular, the lower effective size of the SC population compared with the SI population is consistent with the lower genetic variation observed

compared in it (Chapter I), as well as with the expectation that selfing populations should have a lower $N_e$ than outcrossing population in general (Nordborg and Donnelly 1997; Charlesworth and Pannell 2001; Roze and Rousset 2004). The inferred genetic isolation (absence of ongoing gene flow) between the SC and the SI populations is also to be expected, because inbreeding populations tend to invest less in dispersal (reviewed in Charlesworth and Pannell 2001; Barrett et al. 2014) and thus to be more isolated from one another than outcrossing populations (Hamrick and Godt 1996; Duminil et al. 2009; Barrett et al. 2014; but see below).

*Implications for understanding the loss of SI in* L. cavanillesii

Variation in the compatibility status among populations of *L. cavanillesii* is almost certainly due to the loss of SI rather than its gain (Schoen et al. 1997; Igic et al. 2006; Goldberg and Igić 2008, 2012). We may thus suppose that the loss of SI in the lineage contributing to the SC population (COV) occurred at the time of, or after, its divergence from the SI populations from which it is derived, i.e., at least as recently as about 2,810 generations ago. *L. cavanillesii* is a long-lived perennial plant, with individuals that likely live for at least a decade in age-structured populations. It is difficult to estimate generation time of *L. cavanillesii* accurately, but if we allow for mutations rates up to ten times lower than that assumed in our analysis, the age of SC in the species is likely to be between about 3,000 and 30,000 years. Given the isolation and small size of the populations sampled, especially the SC population, it is likely that they have not persisted at the same sites over all that time. Our inference thus should be seen as one that corresponds to the lineage of plants that perhaps gave rise to the sampled populations by colonization from elsewhere, possibly after the time of divergence that our study points to. Such colonization events would likely have reduced the effective size of the populations we sampled, and may have contributed to the selection of SC and the purging of inbreeding depression in the SC lineage (see below).

To what extent might the relatively recent loss of SI in *L. cavanillesii* account for the differences between the derived SC and the SI populations in terms of patterns of inbreeding depression (Chapter II) and their floral biology (Chapter I)? First, given the inference that several thousand generations separate the origin of SC and the individuals we sampled, it is not too surprising to find that inbreeding depression has been purged. Although putative purging has been observed in many SC species (e.g. Weber et al. 2012; Dart and Eckert 2013) , the dynamics of its evolution in natural populations is poorly known and difficult to assess. However, several studies indicate that purging can be rapid. For instance, Noël et al. (2016) recently showed that substantial purging could occur in a SC snail after only 20 generations of forced selfing. Similarly, Benesh et al. (2014) found that inbreeding depression in the tapeworm *Schistocephalus solidus* was reduced for some traits after only two generations of inbreeding. In both experiments, selfing was enforced, with no possibility of outcrossing, though Noël et al. (2016) included infrequent crossing in their experimental design. In contrast, selfing rates in the SC population of *L. cavanillesii* are substantially lower than 100% (s = 0.59; Chapter I). Nevertheless, even with intermediate selfing rates, which might become high for periods of low pollinator visitation (Chapter IV), it seems likely that sufficient time has elapsed since the origin of SC for the population's genetic load to have been purged (Husband and Schemske 1996; Crnokrak and Barrett 2002). Indeed, the relatively small population size, low genetic diversity and high population isolation of the SC population (Chapter I) are consistent with conditions that might accelerate the purging of deleterious alleles from a population (Kirkpatrick and Jarne 2000; Pujol et al. 2009; Pekkala et al. 2012; Oakley and Winn 2012; Lohr and Haag 2015; Hedrick and Garcia-Dorado 2016).

Despite the likely rapid purging of inbreeding depression, the SC population shows no evidence for the evolution of a selfing syndrome (Chapter I), i.e., a reduction in pollen production or of allocation towards attracting and rewarding pollinators (Ornduff 1969; Sicard and Lenhard 2011). One explanation is that the evolution of a selfing syndrome simply necessitates more time than is required for the purging of inbreeding depression, and that 3,000 generations have been insufficient. In their study using the

hermaphrodite freshwater snail *Physa acuta*, Noël et al (2016) found that although early-acting inbreeding depression was purged rapidly, male allocation remained unchanged over the 20-generation course of their experiment. In contrast, Bodbyl Roels and Kelly (2011) have shown that morphological changes relevant to a selfing syndrome can evolve within a few generations if there is sufficient variation upon which selection can act, a finding consistent with what is known more generally about the capacity of populations under natural selection to undergo rapid morphological changes (e.g. Seeley 1986; Losos et al. 1997; Reznick et al. 1997; Lendvai and Levin 2003). It is also plausible that genetic variation in the SC lineage of *L. cavanillesii* was low, e.g., as a result of population bottlenecks, and that it was thus unresponsive to selection.

Alternatively, it is possible that selection has maintained an outcrossing syndrome in the SC lineage of *L. cavanillesii*. The SC population currently shows mixed mating (with a selfing rate of about 0.6; Chapter I), and plants frequently attract and reward pollinators. Other studies that have investigated the divergence time among populations with contrasting mating systems have also found that relatively recent transition are not associated with a selfing syndrome (e.g. Foxe et al. 2010; Busch et al. 2011). For instance, in SC populations of *Arabidopsis lyrata*, the loss of SI occurred an estimated 10,000 years ago, and flowers still show an outcrossing syndrome (Hoebe et al. 2009; Foxe et al. 2010). Similarly, in *Leavenworthia alabamica*, a population that evolved SC ~48,000 years ago displays only slight changes in floral size and pollen production (Busch et al. 2011). These cases contrast with that of *Capsella rubella*, where a strong selfing syndrome has evolved in less than an estimated 20,000 years (Foxe et al. 2009; Slotte et al. 2012).

On balance, the idea that the SC lineage of *L. cavanillesii* has been unresponsive to selection for increased selfing and reduced allocation towards pollinator attraction and reward for thousands of generations seems to us implausible, but observations in other species referred to above clearly present a similar conundrum. Given that inbreeding depression has evidently been purged from the SC lineage of *L. cavanillesii* (Chapter II), it is difficult to imagine the basis of selection to maintain outcrossing and an

outcrossing syndrome in this particular case. It remains possible that SC has evolved even more recently than the divergence time we have inferred for the split between the COV lineate and the SI lineages sampled, and that the transition was facilitated by a severe population bottleneck that also removed genetic variation upon which selection for increased selfing might otherwise have acted.

## ACKNOWLEDGMENTS

We thank funding from the University of Lausanne to JRP in support of a studentship to MV. CR was supported by funds to JRP from the Swiss National Science Foundation.

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**Table 1**. **Model comparison between Model I (isolation model) and Model IM (on-going migration model) using ABC simulations for three pairs of *Linaria cavanillesii* populations (COV, BER, DEN).** Populations 1 and 2 refer to the population pair for which the corresponding models were compared. Incompatibility status is indicated in parenthesis for each population (self-compatible: SC vs. self-incompatible: SI)

| Population 1 | Population 2 | Model I | Model IM | Robustness |
|---|---|---|---|---|
| COV (SC) | DEN (SI) | 0,938 | 0,062 | 1 |
| COV (SC) | BER (SI) | 0,657 | 0,343 | 1 |
| BER (SI) | DEN (SI) | 0,09 | 0,91 | 1 |

**Figure 1**. **Geographical range of *Linaria cavanillesii* in Spain (grey oval), and the populations that have been studied in detail (Chapter I)**. Three northern populations (COV, DEN and BER) were used in the ABC simulations presented here. Their self-incompatibility status (SC: self-compatible; SI: self-incompatible) revealed by controlled crosses is indicated.

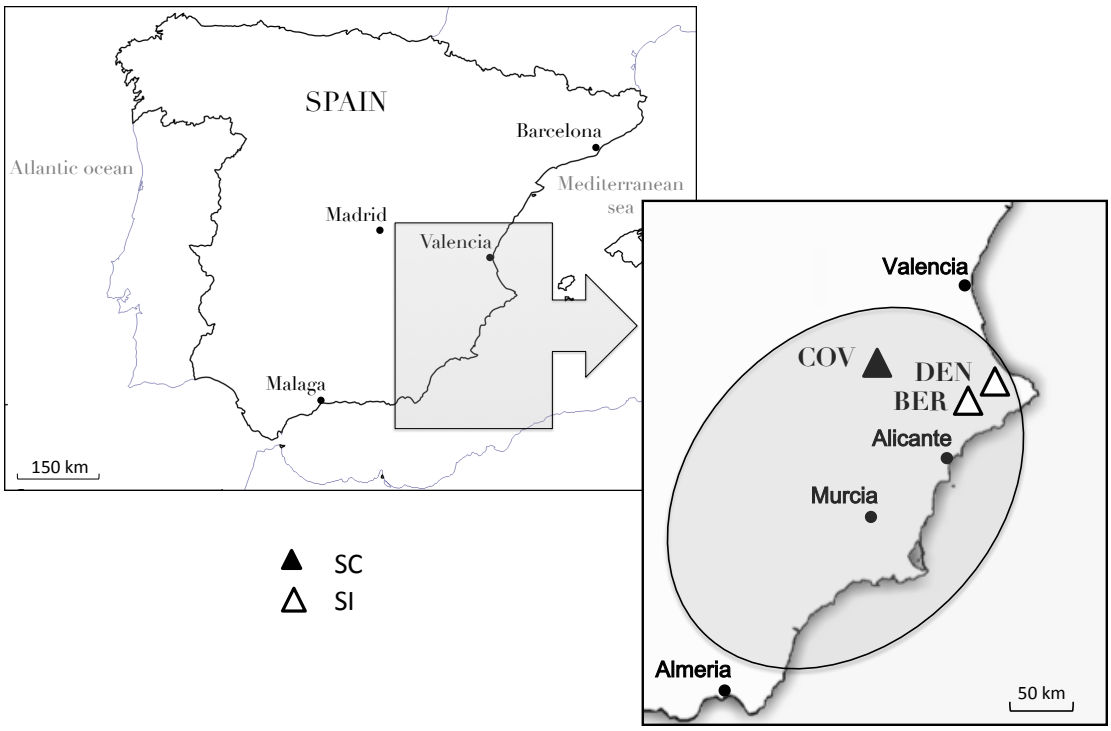


**Figure 2. Illustration of the two scenarios compared by ABC for the history of divergence among *Linaria cavanillesii* populations.** I model (isolation model); IM model (isolation and migration model). $N_1$, $N_2$ and $N_{anc}$ are the number of effective individuals for each population. $T_{split}$ is expressed in generations and represents the time since the ancestral subdivision.

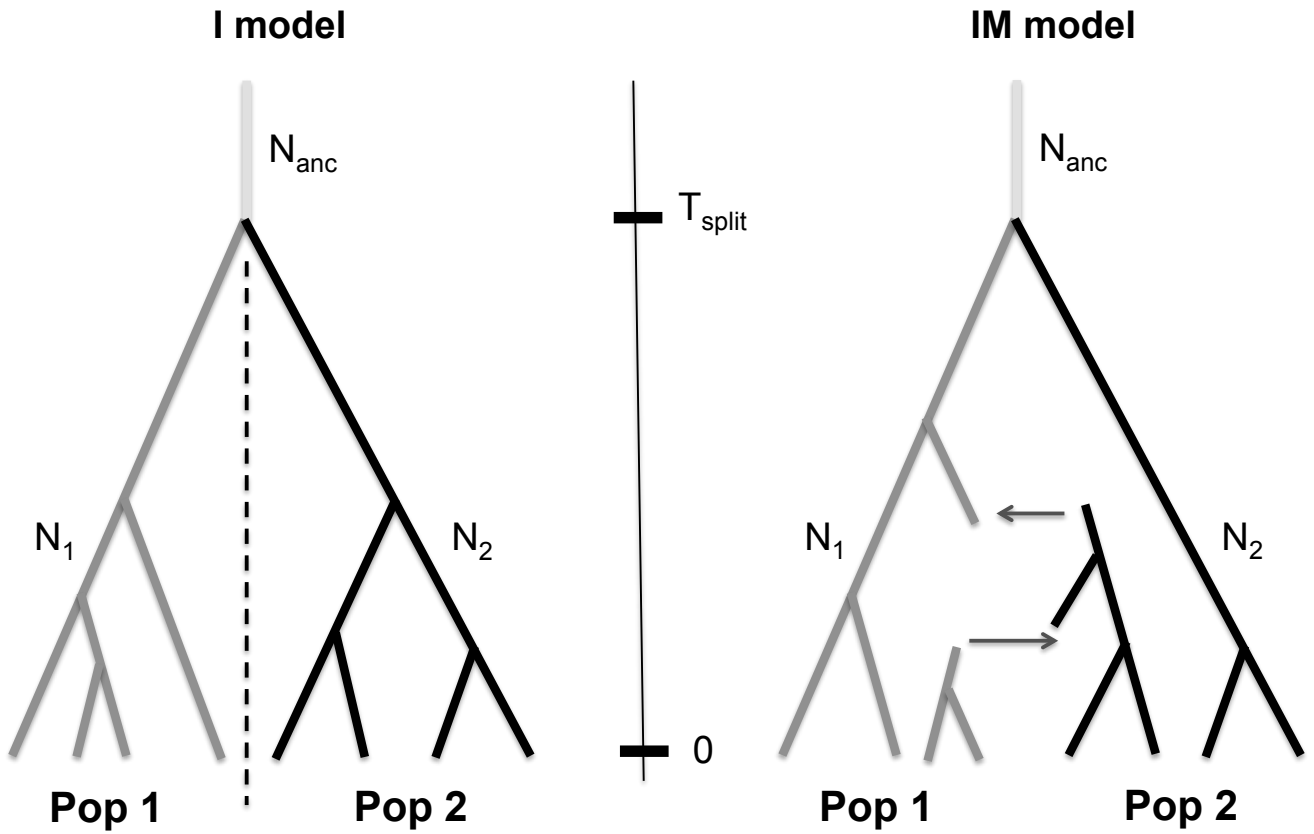
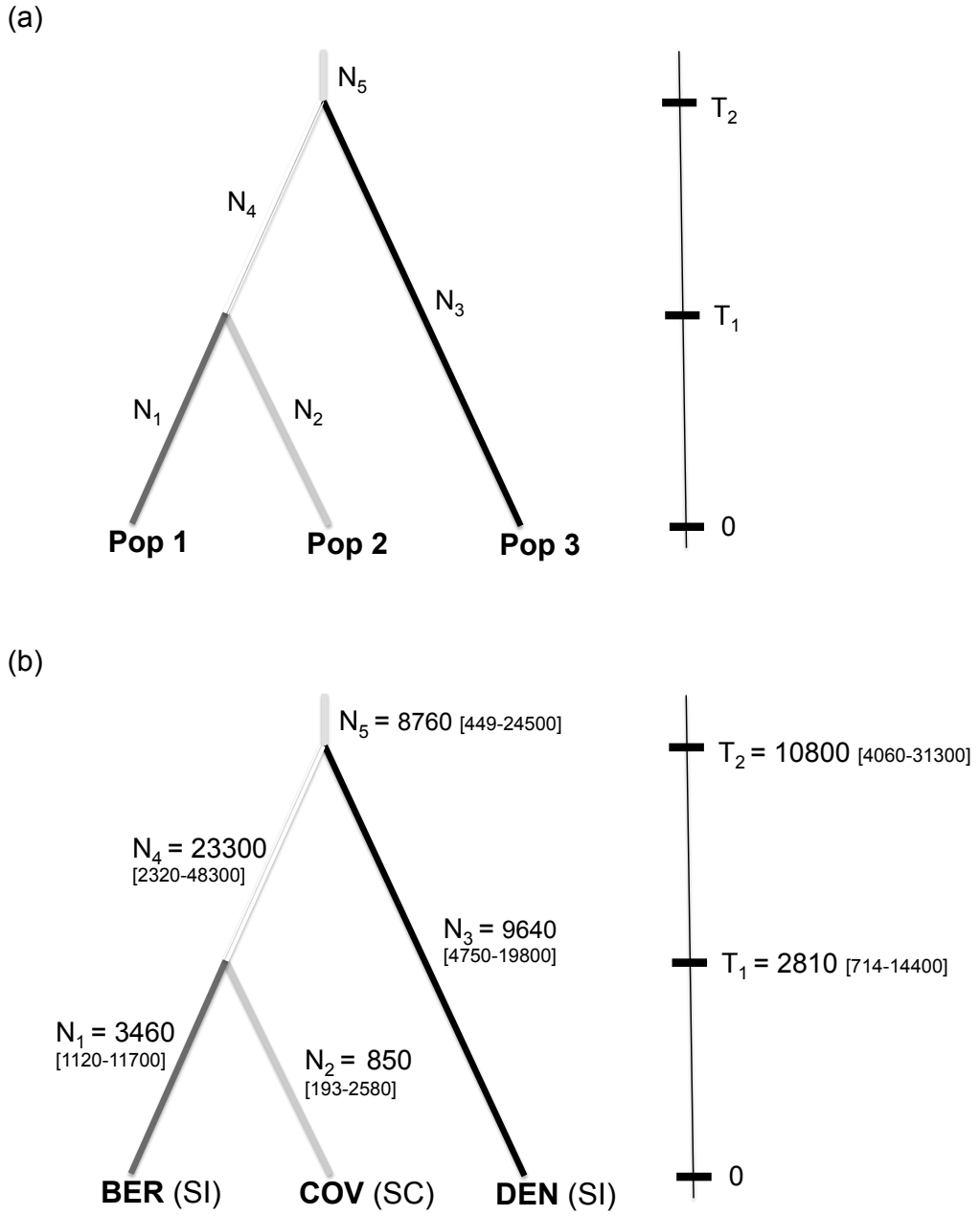


**Figure 3. (a) Illustration of competing scenarios of divergence for three populations of *Linaria cavanillesii*.** We tested scenario 1: pop1 = DEN, pop2 = BER, pop3 = COV; scenario 2: pop1 = DEN, pop2 = COV, pop3 = BER; and scenario 3: pop1 = BER, pop2 = COV, pop3 = DEN. **(b) Parameter estimation based on the best-supported scenario (scenario 3)**. $N_1$, $N_2$, $N_3$, $N_4$ and $N_5$ are the number of effective individuals in the respective populations; $T_1$ and $T_2$ are expressed in generations and represent the time of the ancestral subdivision. For each parameter estimate, 95% CI intervals are indicated in square brackets. The population incompatibility status is indicated: BER and DEN are self-incompatible (SI); COV is self-compatible (SC). See text for details.



# SUPPLEMENTARY INFORMATION

**Table S1. Summary statistics from the ABC simulations**. NAL : number of alleles, HET : genic diversity, VAR : size variance, MGW : Garza Williamson's, $F_{ST}$, LIK : classification index, DAS : shared allelic distance between populations, DM: $(d\mu)^2$ distance; observed value (Obs.value) and proportion of simulated value < observed value among 1,000 simulations (prop. (sim < obs).

| | Summary statistics | Obs. value | Prop. (sim<obs) |
|---|---|---|---|
| **One sample stat.** | NAL_1_1 | 1.50 | 0.51 |
| | NAL_1_2 | 2.19 | 0.31 |
| | NAL_1_3 | 2.94 | 0.36 |
| | HET_1_1 | 0.09 | 0.37 |
| | HET_1_2 | 0.34 | 0.58 |
| | HET_1_3 | 0.27 | 0.12 |
| | VAR_1_1 | 0.09 | 0.30 |
| | VAR_1_2 | 1.12 | 0.71 |
| **Two sample stat.** | VAR_1_3 | 1.15 | 0.64 |
| | MGW_1_1 | 0.89 | 0.57 |
| | MGW_1_2 | 0.69 | 0.16 |
| | MGW_1_3 | 0.73 | 0.20 |
| | N2P_1_1&2 | 2.50 | 0.26 |
| | N2P_1_1&3 | 3.19 | 0.29 |
| | N2P_1_2&3 | 3.38 | 0.18 |
| | H2P_1_1&2 | 0.31 | 0.46 |
| | H2P_1_1&3 | 0.34 | 0.28 |
| | H2P_1_2&3 | 0.42 | 0.39 |
| | V2P_1_1&2 | 0.71 | 0.47 |
| | V2P_1_1&3 | 0.99 | 0.51 |
| | V2P_1_2&3 | 1.40 | 0.63 |
| | $F_{ST}$_1_1&2 | 0.56 | 0.62 |
| | $F_{ST}$_1_1&3 | 0.63 | 0.86 |
| | $F_{ST}$_1_2&3 | 0.44 | 0.83 |

| | | |
|---|---|---|
| LIK_1_1&2 | 0.97 | 0.42 |
| LIK_1_1&3 | 1.39 | 0.56 |
| LIK_1_2&1 | 1.56 | 0.47 |
| LIK_1_2&3 | 1.33 | 0.51 |
| LIK_1_3&1 | 1.74 | 0.34 |
| LIK_1_3&2 | 1.28 | 0.38 |
| DAS_1_1&2 | 0.53 | 0.47 |
| DAS_1_1&3 | 0.50 | 0.59 |
| DAS_1_2&3 | 0.46 | 0.45 |
| DM2_1_1&2 | 0.87 | 0.46 |
| DM2_1_1&3 | 1.48 | 0.55 |
| DM2_1_2&3 | 1.09 | 0.58 |

**Figure S1. Posterior probability for the time of a split between populations COV and BER (red line), or between DEN and the combined pair COV-BER (blue line).**

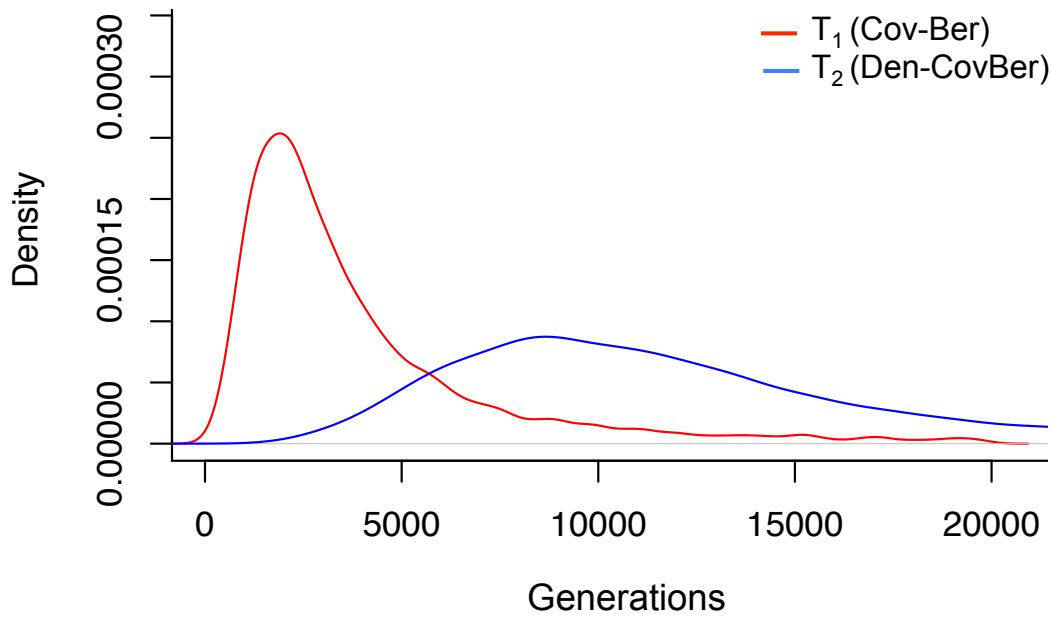


**Figure S2. Distributions of estimated posterior probabilities.** Each distribution was obtained by ABC analysis of 300,000 simulated pseudo-observed datasets using the software DIYABC. The area under each curve represents the probability that the chosen model (scenario 3) is correctly assigned by our procedure. M1: scenario 1, M2: scenario 2, M3: scenario 3.

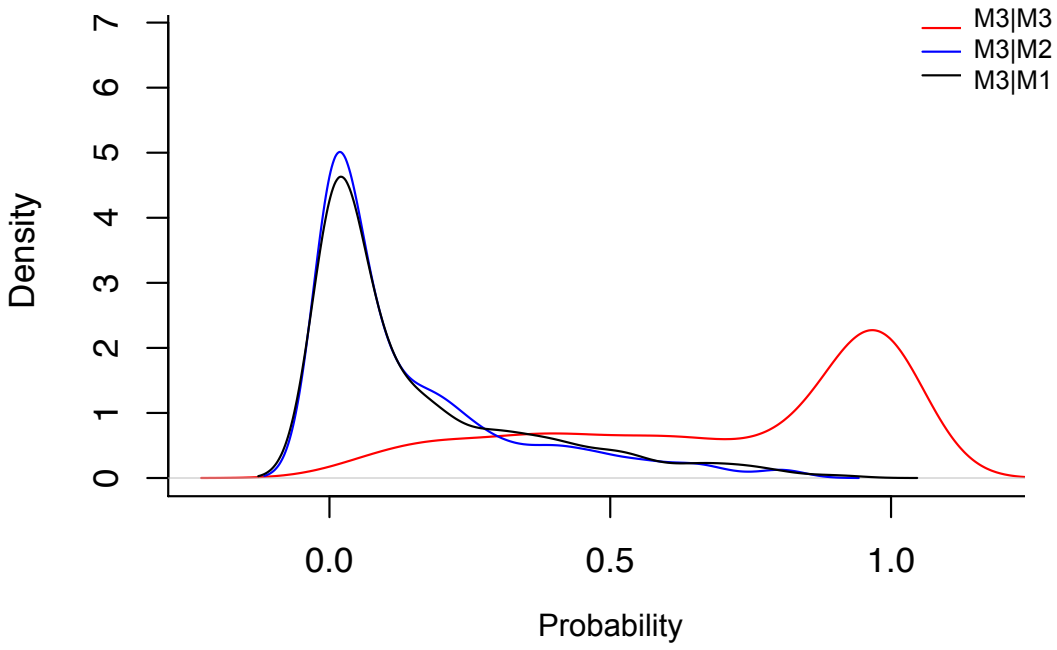
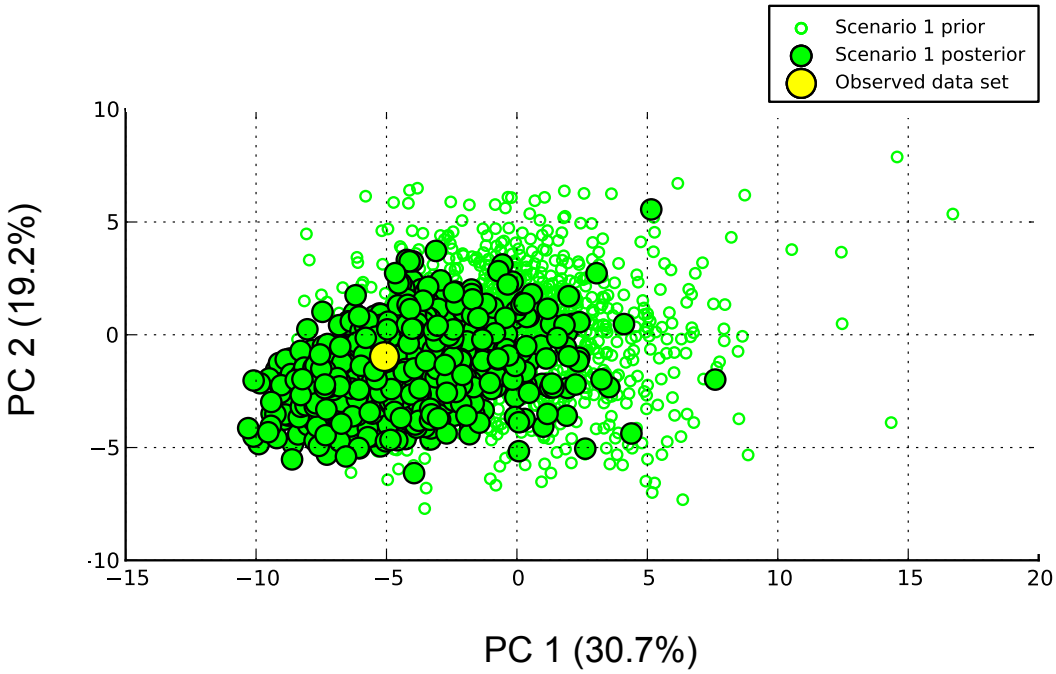


**Figure S3. Principal component analysis of summary statistics using 1,000 data sets simulated with the prior distributions for the chosen parameters, the observed data and data from the posterior predictive distribution.**



# - CHAPTER IV -

## A rapid transition to self-compatibility in experimental populations of the self-incompatible herb, *Linaria cavanillesii*

M. Voillemot and J. R. Pannell

*(In preparation)*

# ABSTRACT

The evolution of increased self-fertilization is one of the most frequent transitions to have taken place in flowering plants, but our understanding of its causes, mechanisms and consequences remains incomplete. Specifically, it is still not clear how self-incompatible (SI) and self-compatible (SC) plants interact with each other when competing in similar environments, and how one strategy might spread from one population into others after its initial establishment. Here, we report on a multi-generation experiment with experimental populations of the perennial insect-pollinated plant *Linaria cavanillesii*, which shows inter-population variation in SI. We estimated components of fitness for SC and SI individuals over three successive generations in six experimental populations that differed in their exposure to pollinators, with all arrays starting with equal frequencies of SC and SI plants. A complementary experiment considered the spread of SC from a lower starting frequency. We observed a rapid shift toward SC in all arrays of both experiments, especially when pollinators were sparse or absent. The success of SC in all arrays was due to both higher fruit set in SC individuals, but, surprisingly, also to greater outcross siring success. Our experiments also suggest that, in the short term, even a moderate presence of pollinators may be sufficient to maintain mixed mating in natural conditions in this species. We discuss the long-term implications of the apparent superiority of SC over SI in *L. cavanillesii* and the broader implications of our results for our understanding of how SC may introgress into SI populations, an ultimate prerequisite to the species-wide fixation of SC in flowering plant lineages.

**Keywords**: fruit set, mating system, outcrossing rate, paternity, pollen discounting, pollinator, reproductive assurance, selection experiment, selfing rate

# INTRODUCTION

An estimated 40% of angiosperm species are hermaphrodites that possess a self-incompatibility (SI) system, which prevents pollen from fertilizing ovules of the same plant and thus promotes outcrossing. Although individuals in SI species benefit from avoiding the negative fitness consequences of inbreeding depression in their progeny (Charlesworth and Charlesworth 1987), and SI species are less prone to extinction than those that are self-compatible (SC) (Goldberg et al. 2010), SC has evolved frequently in angiosperms. Indeed, the evolution of SC from SI represents perhaps the most frequent evolutionary transition to have taken place in flowering plants (Stebbins 1974; Igic et al. 2008). Why SI has broken down in some species, and what maintains SI in the face of frequent mutations to SC in others, has been the focus of substantial theoretical and empirical study (Cheptou 2012; Goldberg and Igić 2012; Shimizu and Tsuchimatsu 2015).

Two broad hypotheses have been proposed to explain transitions from outcrossing to self-fertilization, which the breakdown of SI first allows. First, as Fisher (1941) pointed out, individuals capable of self-fertilization should have an immediate gene transmission advantage over individuals in the same population that can only outcross, because each selfed seed carries two copies of the plant producing them (one from the ovule, and one from the pollen grain that sired it), whereas outcrossed seeds carry only one copy (contributed by the ovule). All else being equal, selfing should confer 50% greater fitness than outcrossing, and mutations that increase the selfing rate should spread automatically in a population. The fact that SI is so commonly maintained in plant populations indicates that these conditions probably do not often occur in SI populations, i.e., because selfed progeny suffer from inbreeding depression, and selfing compromises outcrossing opportunities through pollen discounting.

First, selfed progeny will often suffer reduced viability and/or fertility as a result of inbreeding depression (Charlesworth and Charlesworth 1987). In general, if inbreeding depression reduces the fitness of selfed

progeny by more than 0.5 relative to outcrossed progeny, mutations that increase the selfing rate will be deleterious and selected against (Lande and Schemske 1985). This threshold increases for mutations that cause a loss of self-incompatibility, i.e., SC individuals may invade and spread in SI populations when inbreeding depression is higher than 0.5. This is because mutations that confer SC can be transmitted not only though self-fertilization, but also because they render pollen more frequently compatible with other SI individuals in the population that might otherwise have shared S-alleles (Wright 1939; Vallejo‐Marín and Uyenoyama 2004; Layman et al. 2017). Inbreeding depression is nevertheless often sufficiently high in outcrossing populations (e.g., Sletvold et al. 2013; Chapter II) to constitute a major reason for the maintenance of SI in many species. Inbreeding depression is a particularly important barrier to the evolution of increased selfing if self-fertilization involves seed discounting, i.e., if selfing removes ovules from the pool that might have been outcrossed (Lloyd 1992; Herlihy and Eckert 2002).

Second, although mutations conferring SC can increase an individual's outcross siring success for the reasons just outlined (Layman et al. 2017), it may also reduce success as a sire if there is pollen discounting (Nagylaki 1976; Harder and Wilson 1998). In this case, the automatic genetic transmission advantages of selfing are diminished so that mutations for increased selfing are less likely to spread (Porcher and Lande 2005). Pollen discounting can occur because pollen used up in self-fertilization becomes unavailable for outcrossing, e.g., where selfing is the result of pollen transfer between different flowers of the same individual (i.e., geitonogamous self-pollination; Harder and Barrett 1995). Self-fertilizing individuals may also be poorer sires if they are less attractive to pollinators than outcrossing individuals, e.g., if they have smaller flowers, smaller inflorescences. Although many habitually self-fertilizing plants have a 'selfing syndrome', with smaller flowers, less nectar and lower pollen:ovule ratios (thus being less attractive to pollinators), mutations with pleiotropic effects that both increase the selfing rate and a plant's attractiveness to pollinators seem unlikely. By contrast, if self-fertilizing plants produce fewer fruits (e.g., because of early-acting inbreeding depression), they might produce more flowers and thus be *more* attractive to pollinators, potentially leading to the equivalent of negative pollen discounting

(e.g., Stone et al. 2014). Negative pollen discounting should make the evolution of self-fertilization more likely.

Although an automatic transmission advantage must always be taken into account when predicting whether self-fertilization should evolve, a capacity to produce progeny by self-fertilization may confer an additional advantage of reproductive assurance in situations where mates or pollinators are absent or scarce (Darwin 1876; Jain 1976; Eckert et al. 2006; Layman et al. 2017). Indeed, the advantage of reproductive assurance is probably critical to explain transitions from outcrossing to selfing in species in which inbreeding depression is initially high. Once selfing evolves under selection for reproductive assurance, inbreeding depression should often be purged from populations (Barrett and Charlesworth 1991; Crnokrak and Barrett 2002; Dart and Eckert 2013; Benesh et al. 2014), so that reversion to outcrossing is then unlikely, even when pollinators or mates become abundant again. Thus, a period during which mates or pollinators are scarce is thought to be a key reason for many of the frequent transitions from outcrossing to selfing in angiosperms (Kalisz and Vogler 2004; Moeller and Geber 2005; Eckert et al. 2006; Busch and Delph 2012). Another possible cause is a reduction in inbreeding depression brought about by population bottlenecks, e.g., during species range expansions (Pujol et al. 2009; Foxe et al. 2010). Both selection for reproductive assurance, and/or selection under reduced inbreeding depression following population bottlenecks, provide plausible explanations for the increased frequency of selfing and SC in geographically marginal populations of a species range (Baker 1955; Sakazono et al. 2012; Grossenbacher et al. 2015; Pannell 2015; Pannell et al. 2015).

It seems well established that selfing and SC are more likely to evolve in either environmentally or geographically marginal populations (Baker 1955; Grossenbacher et al. 2015; Pannell 2015), but the ultimate fate of mutations conferring an ability to self in such populations is not so clear. Three possible scenarios can be envisaged. Either (1) the species persists with both outcrossing (SI) populations in the core of its range and partially selfing (SC) populations at its margins; (2) the capacity to self spreads from

the marginal populations into the species' core range, bringing about a species-wide transition from outcrossing to selfing; or (3) obligate outcrossing invades the marginal populations, so that self-fertilization is only transient. Conditions for the invasion and spread of SC have been the focus of intensive study from both a theoretical and empirical perspective (e.g., Fisher 1941; Lloyd 1979; Lande and Schemske 1985; Schoen et al. 1996; Barrett 2003; Eckert et al. 2006), including studies that have directly competed SC and SI populations in experimental mating arrays (Stone et al. 2014; Layman et al. 2017), but little research has considered how selfing might spread, or be displaced, across a species' range after it evolves in a subset of populations. This is an important lacuna, because the conditions for a mutation conferring SC to spread within a SI population carrying substantial genetic load of deleterious mutations, as recently explored in elegant experiments by Stone et al. (2014) and Layman et al. (2017), will likely differ from the species-wide fate of SC drawn from a population in which inbreeding depression has been purged.

Here, we evaluate the fate of self-compatibility when individuals sampled from a completely SC population from which inbreeding depression has been purged are introduced into populations comprising SI individuals sampled from the species' core in which there is substantial inbreeding depression. Specifically, we established replicated mixed-array populations of the perennial plant *Linaria cavanillesii* with SC and SI individuals under different levels of exposure to pollinators, and we measured the frequency of the two compatibility morphs, their selfing rates and male and female components of their fitness over three successive generations. Our experiment allowed us to determine how differences in reproductive assurance, selfing rates, flower, fruit and seed production, effective pollen discounting and siring success actually translate into changes in the composition of each experimental population, and to interpret the latter outcome in terms of the former processes.

Recently, Stone et al. (2014) estimated the fitness components, selfing rates and pollen discounting in mixed experimental populations of the perennial shrub *Witheringia solanacea*. In another recent study,

Layman et al. (2017) followed the early fate of an SC allele that they had experimentally introgressed into an SI background of the species *Leavenworthia alabamica*. Both studies report that SC conferred greater siring success (though not significantly so in *L. alabamica*), and both assess the importance of reproductive assurance, inbreeding depression and pollen discounting for the spread of SC. We too find that SC individuals are more successful sires than SI individuals in *Linaria cavanillesii*, although dramatically more so than documented in these other two studies. To our knowledge, our study is the first study to evaluate the trajectory of SC as it spreads in the population over successive generations under conditions where pollinator availability were manipulated experimentally.

# MATERIAL AND METHODS

*Study species*

*Linaria cavanillesii* is a long-lived perennial herb that grows on north-northwest-facing limestone cliffs in southeastern Spain. Plants flower in April through June, producing large leafy inflorescences composed of yellow flowers with long nectar spurs that are visited mainly by bees and bumblebees (Chapter I). Fruits typically mature approximately 30 days after fertilization, and seeds are dispersed passively when capsules open. Based on the study of one of its populations, the species was until recently thought to be SC (Carrió et al. 2013), but research has now shown it to be largely SI, with only a single SC population known, although several population show a small degree of 'leaky' SI (Chapter I).

SC individuals of *L. cavanillesii* have flowers of the same size as SI individuals and produce similar nectar of a similar quantity and quality; there is thus no indication that the evolution of SC has coincided with a selfing syndrome (Chapter I). By contrast, while the SI populations of *L. cavanillesii* show substantial inbreeding depression upon selfing, there is no difference in the fitness of selfed and outcrossed progeny produced by SC mothers (Chapter II). It seems likely that *L. cavanillesii* evolved SC

recently, and that although inbreeding depression has been purged from the SC population, the floral biology of the species has not changed in the wake of the transition to SC. Accordingly, pollinator visitation of SC individuals in the field remains high, and the population shows mixed mating as a result, with intermediate outcrossing rates (Chapter I). The current study was carried out using seeds two SI populations (BER and DEN) and the single known SC population (COV), all from the Alicante region in southeastern Spain (Figure S1).

*Experimental design*

Our study comprised two complementary experiments in which SC and SI plants were established together in replicated populations. In the first, principle, experiment (experiment 1), we established two experimental populations at each of three levels of pollinator availability, with initially 12 SC and 12 SI plants in each. Access to pollinators was manipulated by enclosing all populations in a netted cage, and by opening the sides of the cage for three different periods of time: open-pollination (plants open to pollination at all times); restricted-pollination (plants open to pollination for two hours three times a week); and no-pollination (total pollinator exclusion) (Figure 1a). Experiment 1 continued for three generations, with genotypes for generation $t$ sampled representatively from seeds produced by individuals in generation $t - 1$, as explained below.

Experiment 1 revealed a rapid increase in frequency in SC plants in all arrays. We thus set up a second, complementary experiment (experiment 2), to determine whether the SC phenotype would enjoy a similar advantage when established at an initially lower frequency. In experiment 2, we thus established 5 SC and 19 SI plants in each of two replicate arrays, both under the open-pollination treatment, though also with the sham netting for comparison with experiment 1 (Figure 1b).

*Passage from one generation to the next*

We used the progeny from generation $t - 1$ to establish new arrays the following year, for generation $t$. Sampling of seeds was random, except that we respected the proportion of fruits produced by SC vs. SI plants. Accordingly, or instance, open-pollination arrays in generation 2 used 15 seeds from SC plants and 9 seeds from SI plants, while restricted-pollination arrays used 14 seeds from SC plants and 10 seeds from SI plants. A similar procedure was adopted for passage from generation 2 to 3. After seeds germinated, we transplanted seedlings into individual pots in which they grew to maturity in the glasshouse before being transferred to their respective plots outside. Compatibility phenotypes were determined for each plant on the basis of controlled crosses. Unfortunately, due to poor seedling germination, we were only able to establish a single replicate for the restricted-pollination treatment in experiment 1 (i.e., generation 2 comprised two replicates for the open-pollination and one replicate for the restricted-pollination treatment; Figure 1a). Because only SC plants were able to produce seeds in generation 1 in the no-pollination treatment, we discontinued this treatment for generations 2 and 3.

*Assessment of female components of fitness*

For each generation in both experiments 1 and 2, we assessed female reproductive fitness on the basis of the average flower and fruit production per plant (fruit set) over the entire experimental period. To do this, we marked and counted newly opened flowers weekly, as well as the number of developing fruits. When large enough, we used rubber glue to seal developing fruits in order to avoid seed loss through spontaneous dispersal from open capsules. Additionally, for the first generation of experiment 1, we measured seed set for all fruit collected over the course of two sampling weeks (N = 518).

*Assessment of male components of fitness and selfing rates*

We used microsatellite markers to determine the sire of a sample of progeny produced during generation 1, with DNA extracted from progeny leaves after germination and culture in a glasshouse. On average, we assayed progeny from six SC and seven SI maternal plants from each treatment and replicate, with one to seven fruits per plant, and an average of six seeds per fruit (Table S1). Our paternity assessment for the main experiment was thus based on sampling a total of 305 SC and 156 SI seeds for the open-pollination treatment (Figure 3a), 66 SC and nine SI seeds for the restricted-pollination treatment (Figure 3b), and 14 SC seeds for the no-pollination treatment (to verify that indeed only self-fertilized seed had been produced by SC plants in this treatment). For experiment 2, our analysis was based on sampling a total of 97 SC and 162 SI seeds (Figure 3c). We inferred paternity and selfing rates on the basis of progeny genotyping for 17 polymorphic microsatellites (Chapter I).

We extracted DNA from the leaves of both mothers and their progeny, as well as of all potential fathers in each array. We aimed to sample 24 progeny seeds per mother, but sample sizes often fell short due to difficulties with growing the plants (see Table S1 for a summary of sampling). We extracted DNA with the DNeasy 96 Plant kit (Qiagen). Polymorphic primers (Chapter I) were amplified by PCR (Biometra thermocycler) using the following reagents: 1xPCR mix: 2 ng/µL template DNA, 10x PCR Buffer, 25 mM $MgCl_2$, 5x Q-solution, 2.5 mM dNTP, 0.2 µM of each primer and 0.5 U/µL of Taq DNA polymerase (HotStarTaq®, Qiagen). The thermocycling conditions were 15 min at 95° followed by 32 cycles of 30 sec at the annealing temperature, 30 sec at 72 °C and 30 sec at 95 °C, followed by one cycle of 1 min at the annealing temperature and a final extension of 30 min at 72 °C. PCR products were then sequenced in an ABI3100 sequencer (Applied Biosystems). We used the program Genemapper® to analyze microsatellites data, and estimated paternity using the software COLONY (Jones and Wang 2010). Selfing-rate estimates were based on paternity analyses.

*Assessment of compatibility phenotypes*

We used two approaches to estimate compatibility phenotypes of the progeny. For generation 2 of experiments 1 and 2, paternity analyses allowed us to infer progeny phenotypes on the basis of the fact that all crosses involving either an SC mother or father (or both) in *L. cavanillesii* always yield SC progeny (M. Voillemot, personal observation). We cross-validated this inference on the basis of observations and controlled crosses in the greenhouses, considering plants for which self-fertilization led to successful fruit formation as SC. For generations 3 and 4 of experiment 1, we assessed the compatibility phenotype of the progeny based only on observations for a subsample of the progeny (N = 294 and N = 261 for generations 3 and 4, respectively) (Figure 5).

*Statistical analyses*

We used linear mixed models to investigate differences in flower production between compatibility phenotypes and treatments, using the lme4 package in R (Bates et al. 2014). For analysis of experiment 1, we considered compatibility phenotype and pollination treatment as fixed effects, and block, plant position and maternal identity as random factors. For experiment 2, compatibility phenotype was the only fixed effect. Significance of variables was analysed using a stepwise deletion procedure on the basis of a likelihood ratio, and multiple comparisons of means were performed using the lmerTest package in R (Kuznetsova et al. 2014). To compare flower production or seed production within treatments, similar models were used with compatibility phenotype specified as a fixed effect and plant position and mother identity as random factors. For binomial data (i.e., fruit set or paternity mating phenotype), we used a generalized linear mixed model (glmer) with binomial errors and random factors as specified above, testing differences within treatments by means of post-hoc tests, with the glht function of the multcomp package in R. For paternity analyses, we compared the siring success of SC vs. SI fathers against random mating using either chi-square tests, or Fisher's exact tests for sample sizes < five observations. All analyses were conducted in R (version 3.2.0 or higher, R Core Team 2015).

# RESULTS

Below, we first present results for both experiments 1 and 2, with the effect of our experimental treatments in generation 1 on flower, fruit and seed production of the two mating-type morphs, on their siring success, and on selfing rates. We then present results of the evolution of SC vs. SI phenotypes over the course of three generations in experiment 1 and after the single generation of experiment 2.

*Effect of SI status and pollinator exclusion on flower number*

There were no significant differences in flower production between the two SI populations used in our experiment, as measured over the seven weeks of generation 1 in plots for experiment 1 (lmer: open-pollination: $F_{2,19} = 0.29$, $P = 0.75$; restricted-pollination: $F_{2,37} = 3.25$, $P = 0.05$; no-pollination: $F_{2,24} = 0.69$ , $P = 0.51$). Nor was there any difference between the two SI populations in their overall fruit set (glmer: open-pollination: $\chi^2_2 = 20.9$, $P < 0.001$; post-hoc test BER/DEN: $P = 0.50$; restricted-pollination: $\chi^2_2 = 34.6$, $P < 0.001$; post-hoc test BER/DEN: $P = 0.60$; no-pollination: $\chi^2_2 = 23.0$, $P < 0.001$; post-hoc test BER/DEN: $P = 0.95$). Given the uniformity observed across the two SI populations, we consider all SI plants as a single group in subsequent analysis below.

Analyzed in this way, there were no significant differences in flower production between the SC and the SI plants among pollination treatments in generation 1 of experiment 1 (lmer for compatibility phenotype x pollination treatment interaction: $F_{2,126} = 0.43$, $P = 0.65$; Figure S2). A similar result was obtained for experiment 2 (lmer: $F_{1,41} = 0.96$, $P = 0.33$; Figure S2). Flower production was somewhat lower overall in the restricted-pollinator and no-pollinator treatments than in the open-pollinator treatment, a result perhaps attributable to the higher temperatures (and thus potentially more stressful conditions) experienced by plants growing in permanently closed pollinator exclosures (lmer for pollination treatment: $F_{2,126} = 32.38$, $P < 0.001$). Importantly, however, there were no differences between SC and SI plants in this effect within

each treatment (post-hoc test: open-pollination: $P = 0.80$; restricted-pollination: $P = 0.70$; no-pollination: $P = 0.50$; Figure S2).

*Effect of SI status and pollinator exclusion on fruit set*

Over the seven weeks of generation 1 of experiment 1, fruit set varied significantly among pollination treatments, as well as between SI and SC mothers (glmer for compatibility phenotype x pollination treatment interaction: $\chi^2_2 = 70.9$, $P < 0.001$; Figure 2a). Notwithstanding this significant interaction, SC plants always had greater fruit set than SI plants within each pollination treatment (glmer: open-pollination$_1$: $\chi^2_1 = 20.5$, $P < 0.001$; restricted-pollination: $\chi^2 = 33.2$, df = 1, $P < 0.001$; no-pollination: $\chi^2_1 = 20.6$, $P < 0.001$; Figure 2a). This trend was similar in experiment 2 (glmer: $\chi^2_1 = 4.25$, $P = 0.04$; Figure 2b). The greater fruit set in SC plants was enhanced with pollinator limitation (open-pollination: 23% higher mean fruit set for SC plants; restricted-pollination: 54% higher mean fruit set for SC plants; no-pollination: 42% higher mean fruit set for SC plants; Figure 2a). As expected, SI plants produced no fruits in the absence of pollinators.

Note that there were no differences in the average number of seeds produced per fruit between SC and SI mothers across pollination treatments in generation 1, experiment 1 (lmer for compatibility phenotype x pollination treatment interaction: $F_{2,340} = 2.06$, $P = 0.13$; post-hoc test for compatibility phenotype: open-pollination: $P = 0.51$; restricted-pollination: $P = 0.30$; no-pollination: $P = 0.15$; Figure S3; we provide no test for plots under the no-pollination treatment, because there were no fruits produced in the total absence of pollinators). We therefore take fruit set to be a reliable estimate of relative female reproductive success in our experimental plots. In contrast with fruit set, there were no differences in the total number of seeds per plant between SI and SC plants under the open-pollination treatment (lmer: open-pollination: $F_{1,46} = 2.31$, $P = 0.14$), but SC plants produced more seeds that SI plants in both the restricted- and the no-

pollination treatments (lmer: restricted-pollination: $F_{1, 42} = 5.34$, $P = 0.03$; no-pollination: $F_{1, 42} = 7.75$, $P = 0.008$; Figure S4). The same trend was observed for fruit set.

As observed in generation 1 of experiment 1, fruit set in generation 2 was again much higher for SC plants compared to SI plants, even if the trend was more pronounced in the restricted-pollination treatment (glmer: open-pollination: $\chi^2_1 = 8.36$, $P = 0.004$; restricted-pollination: : $\chi^2_1 = 74.72$ , $P < 0.001$; Figure S5).

*Effect of SI status and pollinator exclusion on siring success and selfing rates*

Of the total amount of outcrossed seeds produced in the arrays, 87% and 71% of seeds were sired by SC plants in the restricted- and open-pollination treatments, respectively, a result significantly different from random mating (open-pollination: $\chi^2_1 = 16.41$, $P < 0.001$; restricted-pollination: $\chi^2_1 = 8.25$, $P = 0.003$). If we consider SC mothers only in the open-pollination treatment, 71% of the outcrossed seeds were sired by SC fathers and 29% by SI fathers (Figure 3a). The same trend was found for SI plants, where 70% of the outcrossed seeds were sired by SC fathers (Figure 3a). Similarly, in the restricted-pollination treatment, 90% and 78% of the outcrossed seeds were sired by SC fathers, for SC and SI mother plants, respectively (Figure 3b). Although the difference in siring success between SC and SI plants was somewhat smaller in experiment 2, 59% of the total of outcrossed seeds were still sired by SC plants, despite their much lower frequency, a result that fell just short of being statistically different from random mating (chi-square test: $\chi^2_1 = 3.31$, $P = 0.07$). SC fathers thus sired 64% and 57% outcrossed seeds on SC and SI mothers, respectively (Figure 3c).

Selfing rates depended strongly on the pollination treatment, with an average selfing rate of 1.0, 0.68 and 0.41 in the no-pollination (N = 14), restricted-pollination (N = 66) and open-pollination treatments (N =

305), respectively (Figure 4a). In experiment 2, SC plants selfed an average proportion of 0.30 of their progeny (N = 97) (Figure 4b).

*Evolution of SI status over three generations*

Over three generations of mating, we observed a nearly complete shift toward SC in all our arrays (Figure 5a), with the transition being particularly rapid when pollinators were less abundant. As one might expect, only SC plants produced fruits in the no-pollination treatment, so that all plants were SC after a single generation (Figure 5a). In the restricted-pollination treatment, 97% of the progeny were SC after one generation, > 99% after two generations and finally 100% after three generations (Figure 5a). In the open-pollination treatment, 90% of the progeny were SC after one generation, 92% after two generations and > 96% after three generations (Figure 5a). These trends were repeated in experiment 2, with 76% of the progeny being SC after one generation of mating (Figure 5b).

# DISCUSSION

Our study compared male and female components of fitness, selfing rates, and the proportion of compatibility phenotypes of progeny produced by self-compatible (SC) and self-incompatible (SI) individuals in experimental populations of the plant *Linaria cavanillesii* in which we varied pollinator availability. We found that SC individuals produced more seeds and fruits than SI individuals under all three levels of pollinator availability, but particularly when pollinators were scarce or absent. Surprisingly, SC individuals were not only able to self-fertilize a substantial fraction of their ovules, depending on pollinator availability, but they were also substantially more successful as sires of outcrossed ovules in the populations, whether these latter were produced by SC or SI individuals. In accordance with the observed superiority in terms of both female and male components of fitness, we observed the complete fixation of SC in the experimental populations within only three generations of mating. In a second complementary

experiment in which SC plants began as the minority phenotype, we found qualitatively similar results. Below, we first discuss the effects of the experimental treatments on female and male reproductive success and on the mating system. We then consider the implications of our results for an understanding of the maintenance of the compatibility polymorphism found in *Linaria cavanillesii*, as well as of mating-system transitions in plants more generally.

*Pollinator limitation, seed discounting, and reproductive assurance*

Both SI and SC morphs produced similar numbers of flowers, but SC plants produced more fruits per flower than SI plants, while producing similar numbers of seeds per fruit. This pattern suggests that flowers that were visited received sufficient pollen for fruit set in both SC and SI individuals, and that a capacity to self-fertilize increased seed production of SC individuals largely through autonomous seed set in unvisited flowers, a reproductive advantage that was particularly strong when pollinator availability was reduced experimentally. Recall that there is limited separation between anthers and the stigma within flowers of *L. cavanillesii*, so that self-fertilization is effectively automatic and autonomous in SC individuals. This means that prior selfing in flowers that are not visited soon after they open will cause seed discounting, i.e., prior selfing removes ovules from the pool that might otherwise be outcrossed later (e.g., Herlihy and Eckert 2002). For two reasons, however, seed discounting is unlikely to have an important impact on the fitness of SC individuals of *L. cavanillesii*. First, because SC individuals of *L. cavanillesii* show negligible inbreeding depression (Chapter II), such seed discounting should not be costly (though see below). Second, selfing in *L. cavanillesii* does not occur completely prior to outcrossing opportunities, because plants, when exposed to pollinators, outcross a substantial number of their ovules. Indeed, outcrossing rates correlated with pollinator availability. Because seed set in the total absence of pollinators is high, it seems clear that the capacity to self-fertilize of SC individuals should bring reproductive assurance under conditions where pollinators are scares or absent. This capacity

certainly helps to explain the more rapid spread of SC we observed in our experimentally populations from which pollinators were excluded by netting.

*Pollinator-dependent selfing, and the maintenance of an outcrossing syndrome*

As just noted, the capacity for autonomous selfing of SC plants did not preclude outcrossing in experimental populations to which pollinators were allowed access. In particular, whereas all seeds produced by SC plants were self-fertilized in populations from which pollinators were totally excluded, 32% and 59% were outcrossed when pollinators had partial or full access to the populations, respectively. It is thus clear SC individuals of *L. cavanillesii* enjoy complete reproductive assurance in the absence of pollinators, but that outcross pollen competes with self-pollen when pollinators visit flowers, so that mixed mating prevails, with the outcrossing rate depending positively on pollinator visitation. It is also clear that, in *L. cavanillesii*, seed discounting is context dependent, a feature that is not typically accounted for in discussions of the phenomenon (Lloyd 1992; Schoen et al. 1996; Herlihy and Eckert 2002). In particular, when pollinators are abundant, the ability to self-fertilize will not substantially compromise the outcrossing potential of SC plants, whereas, in the absence of pollinators, seed discounting is of course not relevant.

We have recently discussed at length why SC *L. cavanillesii* should continue to invest in pollinator attraction when SC provides reproductive assurance (Chapter I). The maintenance of an outcrossing syndrome in plants that have evolved SC might often be attributable to selection for outcrossing when inbreeding depression persists in populations (Dart et al. 2012; Dart and Eckert 2013), but inbreeding depression appears to have been purged from the selfing population of *L. cavanillesii* in the wild (Chapter II). Rather, it seems likely that an ability to self has evolved very recently in this species, and that there has simply been insufficient time for selection to bring about an emphasis of prior over competing selfing, and to favor a reduction in investment towards pollinator attraction and reward, such as had been found in

other species (Busch 2005; Busch et al. 2011; Carleial et al. 2016). In contrast, inbreeding depression can be purged very quickly from populations, particularly for mutations that affect early stages of the life cycle which we have measured in *L. cavanillesii* (Benesh et al. 2014; Noël et al. 2016).

*Relative siring success of SC and SI plants*

A particularly striking and surprising result of our study is the finding that SC plants had higher siring success than SI plants under all experimental conditions where pollinators were allowed access to the plants. Indeed, considering only outcrossing events, SC plants sired more seeds than SI plants. This pattern of mating points to the equivalent of negative pollen discounting, where partially selfing plants sired more, rather than fewer, outcrossed progeny. Negative pollen discounting is uncommon in plants, but both positive and negative pollen discounting were observed in experimental populations of SC *Iponomea purpurea* comprising plants with different degrees of anther-stigma separation (Chang and Rausher 1998). Similarly, in tristylous *Eichhornia paniculata*, negative pollen discounting was found for a selfing morph under conditions of low morph diversity (Kohn and Barrett 1994). In their study of mixed populations of the perennial shrub *Witheringia solanacea*, Stone et al. (2014) also found that SC individuals were more successful sires than SI individuals. In that study, fruit set in SC plants was substantially lower than in SI plants, probably due to early-acting inbreeding depression and fruit abortion, and flower number was accordingly higher (presumably as a result of allocation trade-offs between fruits and flowers; Charlesworth and Charlesworth 1981). The authors were thus able to attribute the greater siring ability of SC plants to their greater attractiveness to pollinators. In contrast, we recorded no difference in flower production and the size of the floral display between SC and SI plants of *L. cavanillesii*, and SC and SI flowers also produce similar nectar quality and quantity (Chapter I). We also observed no difference in pollinator visitation between the two morphs in the experimental arrays of the present study (data not presented).

In the absence of differences in the attractiveness of SC and SI individuals to pollinators, it is difficult to explain the greater siring success of SC over SI plants in *L. cavanillesii* in our study. One possibility is that the siring success of SI plants was compromised by incompatibilities between different plants, e.g., because sires and potential dams shared the same S-alleles (Charlesworth and Charlesworth 1979; E Porcher and Lande 2005; Young and Pickup 2010; Layman et al. 2017). However, plants in our experiment were selected from two different populations, and we expect that the numbers of S-alleles might have been quite high, so that the probability of sharing common alleles was low. Indeed, in a parallel study, crosses among individuals from the populations sampled here indicates that S-allele diversity is indeed high (Appendix 2). Cross-incompatibility thus seems unlikely to account for the lower siring success of SI individuals.

Alternatively, it is possible that SC plants were better sires than their SI counterparts because their pollen quantity and/or quality were greater. In a previous study, we found that pollen production by SC individuals was in fact lower than, or similar to, that of SI individuals of *L. cavanillesii* (Chapter I), suggesting that our result here cannot be attributed to differences in pollen quantity. Whether pollen from SC individuals was more competitive than from SI individuals is an open question requiring experimental testing, but, if so, our results would appear to run counter to what has been found in most other studies (Brandvain and Haig 2005; Mazer et al. 2010; Austerlitz et al. 2012; Rebernig et al. 2015). Indeed, it is predicted that pollen from selfers should generally be *less* competitive than pollen from outcrossers, not more competitive (Mazer et al. 2010), and this should result in a bias toward fathers that are more outcrossed, in contrast to what we find here. For instance, outcross siring success was greater for individuals competing with self- vs. outcross pollen in *Collinsia heterophylla* (Lankinen et al. 2015). In another recent study, Sorin et al. (2016) found the same siring success for self and outcrossed pollen that was applied simultaneously on stigmas, but a significant excess of outcross progeny when outcross pollen was applied before self pollen, as one might expect. Detailed analysis of crossing experiments is required

to establish whether SC pollen is indeed more competitive in *L. cavanillesii*, as our results suggest it might be.

*Rapid spread of self-compatibility in* Linaria cavanillesii

Both our experiments revealed a rapid spread of the SC phenotype at the expense of SI under all pollinator exposure treatments. As expected, in the no-pollination treatment (where only SC plants can reproduce), only one generation was sufficient to allow a complete transition to SC in the populations concerned. Somewhat more surprisingly, we also found that SC spread to high frequency in populations under the restricted- and open-pollination treatments, where a shift to complete SC was observed after only three generations in experiment 1. It is clear from this study, and from crosses performed under controlled conditions (Chapter I, II and V), that all progeny from crosses between SC and SI individuals are SC, i.e., that the SC-conferring allele is effectively dominant over any functional S-allele. The fixation of SC phenotypes therefore does not imply the fixation of the SC-conferring allele, and it is likely that functional S-alleles continue to segregate in the experimental populations, unexpressed. Consequently, crosses among SC genotypes in later generations might be expected to regenerate SI phenotypes.

The consistency of the rapid spread of SC, and the corresponding demise of SI, in mixed populations in our experiment is striking, but conclusions about the potential for SC to spread from an SC population into large SI populations of *L. cavanillesii* must be tempered by two caveats. First, our experiments involved introducing SC into SI populations at high frequencies, whereas its potential spread from a SC population throughout the species range would likely involve invasion into SI populations at low frequency following migration. To some extent, our experiment 2 addressed this possibility by starting the SC phenotype at a frequency substantially lower than 50%, but its initial frequency was still around 20%, substantially higher than $1/N$, an initial frequency that would be small following migration into a reasonably large SI population.

A second caveat is that our experiments with *L. cavanillesii* involved the trajectories of compatibility phenotypes over only three generations. While the initial fate of a new variant in a population is of course critical to its success, its ultimate fate will depend on how selection on it changes over time. We sampled SC individuals from a population in which inbreeding depression had been purged, and combined them with SI individuals from populations harboring substantial inbreeding depression (Chapter II). Thus, after the first generation of mating in our experiment, neither selfed individuals nor the SC progeny of crosses between SC and SI individuals would not have suffered reduced fitness due to inbreeding depression. In generation 2, SC individuals resulting from a cross between SC and SI parents would begin to express inbreeding depression, but only as a result of homozygosity of alleles from the SI population, but not of those from the SC population (whose genetic load had been purged). The amount of inbreeding depression expressed by selfed progeny in later generations should depend on the initial frequencies of SI and SC individuals in the population (because the load carried by the population derives only from SI individuals). Against the background of this uncertainty, it would be valuable to investigate the trajectory of the SC phenotype using computer simulations parameterized according to our knowledge of mating in *L cavanillesii*. Such simulations would be particularly useful to assess the joint evolution of the mating system and genetic load (and thus inbreeding depression) in the population, which must influence the ultimate long-term stability of the mating strategies that evolve.

*Concluding remarks*

Our study provides one of the first experimental investigations of the spread of SC in the context of an otherwise SI population (c.f. Stone et al. 2014; Layman et al. 2017). The striking finding that SC in *L. cavanillesii* is evidently able to spread so rapidly raises several questions that remain unanswered. First, why should SC individuals be such better sires than their SI counterparts when pollinators are abundant? The possibility that pollen from SC plants might be more competitive than that from SI individuals is intriguing, no least because it would contradict the expectation that selection under outcrossing should in

fact lead to more competitive pollen (Brandvain and Haig 2005). Crosses involving mixtures of pollen from SC and SI individuals will be needed to test this hypothesis (Chapter V).

Second, the spread of SC in an SI population with high inbreeding depression (Chapter II) raises the possibility that the success of SC might in fact be only temporary, and that, when inbreeding depressions begins to be expressed in genotypes in which the SC-conferring allele has introgressed into a genomic background with high genetic load, functional S-alleles will become favored again. As discussed above, such a scenario is likely to depend to a large extent on how many SC individuals with purged inbreeding depression originally invade an SI population. The use of computer simulations that incorporate both variation in SI and the evolution of inbreeding depression explicitly modeled in terms of the segregation and expression of deleterious mutations, would help to resolve the likely long-term evolution of populations such as those we have established in our experiments here (e.g., see Layman et al. 2017).

Third, the maintenance of SI in most populations of *L. cavanillesii* seems somewhat surprising in light or results here, which suggest that SC migrants should be able to spread in those populations. Previous work has shown that populations of *L. cavanillesii* are extremely isolated from one another from a genetic perspective and that gene flow, particularly between the SC and the SI populations has been effectively absent ($0.56 < F_{ST} < 0.75$; Chapter I). It is thus possible that SI has been maintained only because the SI populations have not been invaded by individuals from the SC range, where selfing likely fixed very recently (Chapter III.). Of course, a certain degree of leaky SI has been recorded in some of the SI populations of *L. cavanillesii*, just as the phenomenon is common in many SI species (Levin 1996; Brennan et al. 2005; Crawford et al. 2010; Zhang et al. 2014); one might expect that selfing should be able to evolve in these populations through the spread and enhancement of leaky SC locally. However, mutations conferring leaky SI likely occur within a genomic context that has not yet been purged of its deleterious alleles, so the scenario is not equivalent to the one we have studied here. It is likely that

selection continues to favor SI in the face of its occasional leaky expression as a strategy to avoid inbreeding depression (e.g. Sletvold et al. 2013).

Finally, our results confirm that although SC individuals are capable of full seed set by autonomous selfing in the absence of pollinators, as observed in the field (Chapter I), they both set a substantial proportion of outcrossed seed when pollinators are available and, moreover, continue to act as outcross sires. These observations are the hallmarks of a regime of selection to maintain outcrossing and are not expected in populations in which self-fertilization is favored (Goodwillie et al. 2010), as has been suggested for *Camissoniopsis cheiranthifolia* (Dart et al. 2012) and *Collinsia verna* (Kalisz and Vogler 2003, 2004), where attractiveness of flowers accounts for the maintenance of outcrossing despite an ability of individuals to self (Kalisz and Vogler 2003, 2004; Dart et al. 2012). Indeed, given that the SC population of *L. cavanillesii* has been purged of its inbreeding depression, one ought to expect mutations to be selected that increase the selfing rate, e.g., by bringing about an increased rate of prior selfing over delayed or competing selfing. The continued presence of a largely outcrossing syndrome is probably best explained by the fact that transition to SC has been very recent (e.g. Foxe et al. 2010; Busch et al. 2011). While it is possible that SC was selected during a period of low pollinator activity, we suggest that a more likely scenario is that the SC lineage arose through a severe population bottleneck that might have deprived one or a few individuals of suitable mates and/or to have purged the bottlenecked population of its inbreeding depression (Kirkpatrick and Jarne 2000; e.g. Guo et al. 2009; Foxe et al. 2010), allowing SC to spread.

## ACKOWLEDGMENTS

We wish to thank J. El-Assad and A.P. Machado for assistance with microsatellites analyses; M. Staiff for seed counting; J. Wang for help with the use of the software Colony; and funding from the University of Lausanne to JRP in support of a studentship to MV.

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**Figure 1. (a) Design of the selection experiment conducted over three generations**. Experimental-array plots initially comprised twelve self-compatible (SC) and twelve self-incompatible (SI) plants of *Linaria cavanillesii*. Arrays were replicated twice for each of three treatments (no-, restricted-, and open-pollination). After generation one (Gen. 1), a random subsample of seeds was used to establish plants for generation two (Gen. 2), so that parents contributed to the next generation in proportion to the number of seeds they had produced. (**b) Design of the second experiment conducted over one generation only.** Mixed-array plots comprised five SI and 19 SC plants in each replicate. The proportion of plants with each compatibility phenotype in each array is indicated in the pie charts (SC: white; SI: grey). See text for further details.

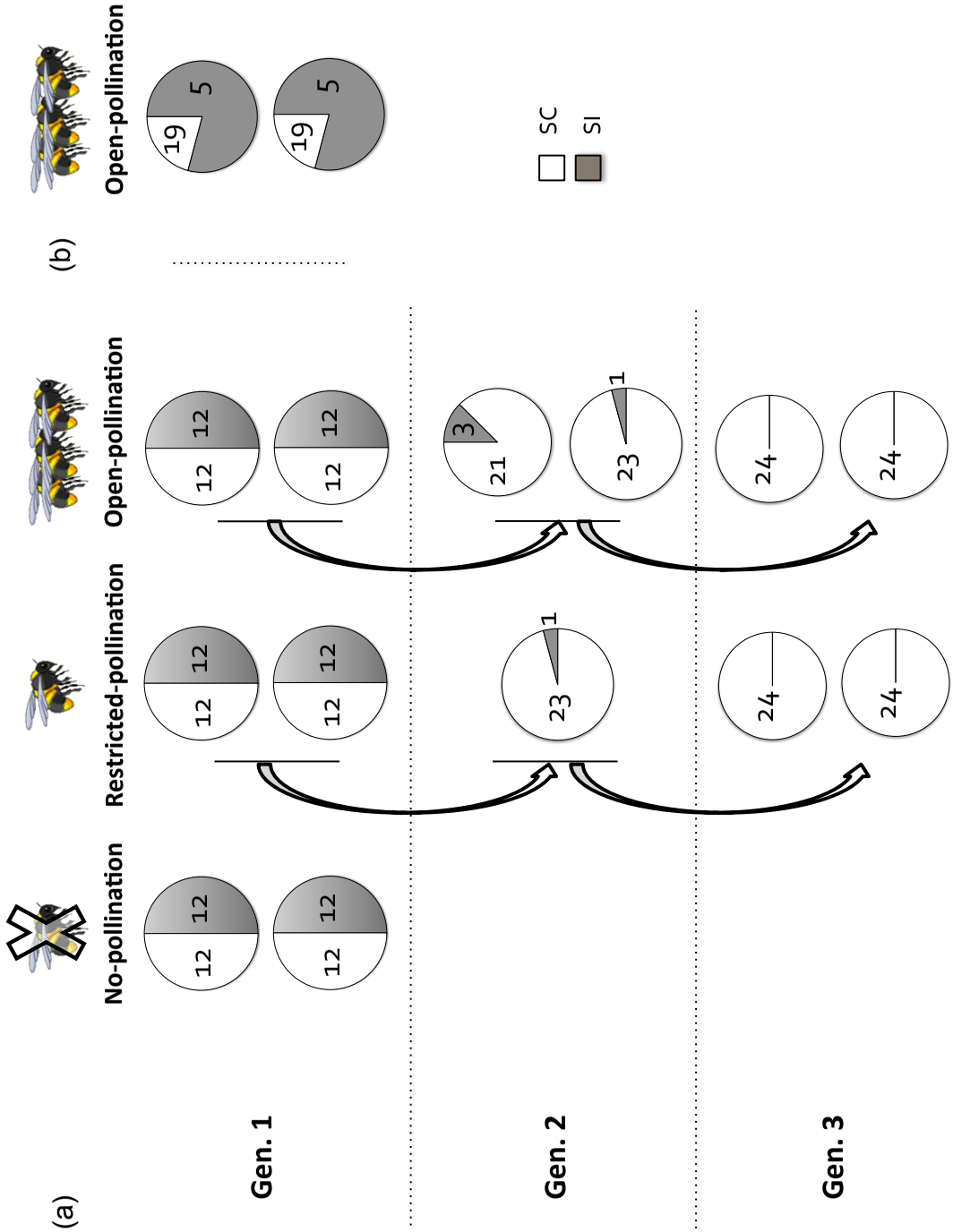

(a)
No-pollination
Restricted-pollination
Open-pollination
Gen. 1
Gen. 2
Gen. 3
12
12
12
12
12
12
12
12
23
1
24
24
3
21
23
1
(b)
Open-pollination
19
5
19
5
SC
SI

**Figure 2. Comparisons of fruit set by self-compatible (SC) and self-incompatible (SI) plants after seven weeks of selection under (a) the open-, restricted-, and no-pollination treatments for plants in generation 1 of experiment 1; and (b) the open-pollination treatment from experiment 2 (see text and Figure 1 for details of the design).** The initial proportions of SC and SI plants are represented by pie charts. Fruit set was calculated as the mean fruit/flower ratio over all plants. In the boxplots, the middle lines represent medians, boxes represent the first and third quartiles, lower and upper bars represent the minimum and the maximum values, and points represent outliers (i.e., points beyond 1.5 standard deviations)**.** Red diamonds represent means. * indicates significant differences between compatibility phenotypes (* : $P < 0.05$; ***: $P < 0.001$).

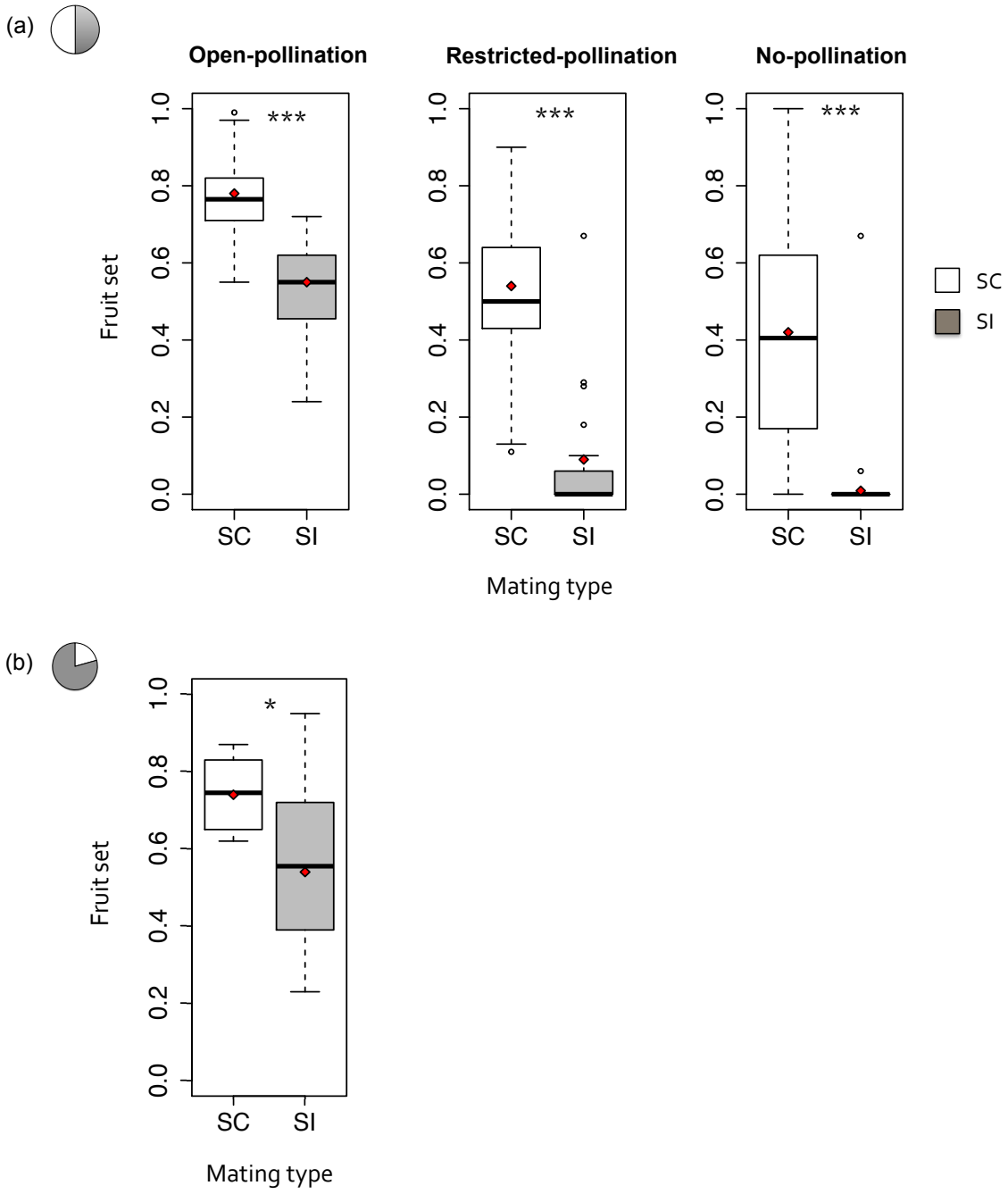


**Figure 3. Comparison of paternity by self-compatible (SC) and self-incompatible (SI) plants after seven weeks of selection under: (a) the open-pollination treatment for plants in generation 1 of experiment 1, (b) the restricted-pollination treatment for plants in generation 1 of experiment 1; and (c) the open-pollination treatment in experiment 2.** Initial proportions of SC and SI plants are represented with the pie charts. Paternity was estimated using microsatellites and the program Colony (Jones and Wang 2010). Bars represent the proportion of selfed seeds (grey), outcrossed seeds sired by SC plants (white) or outcrossed seeds sired by SI plants (dark grey), either when considering all seeds produced ('all seeds') or outcrossed seeds only ('outcrossed seeds'). Number of seeds used for paternity analyses are indicated below the bars. Seeds arising from the no-pollination treatment were 100% selfed, as expected; data not shown. * indicates significant differences from random mating, calculated with a chi-square or Fisher's exact test (see text for details).

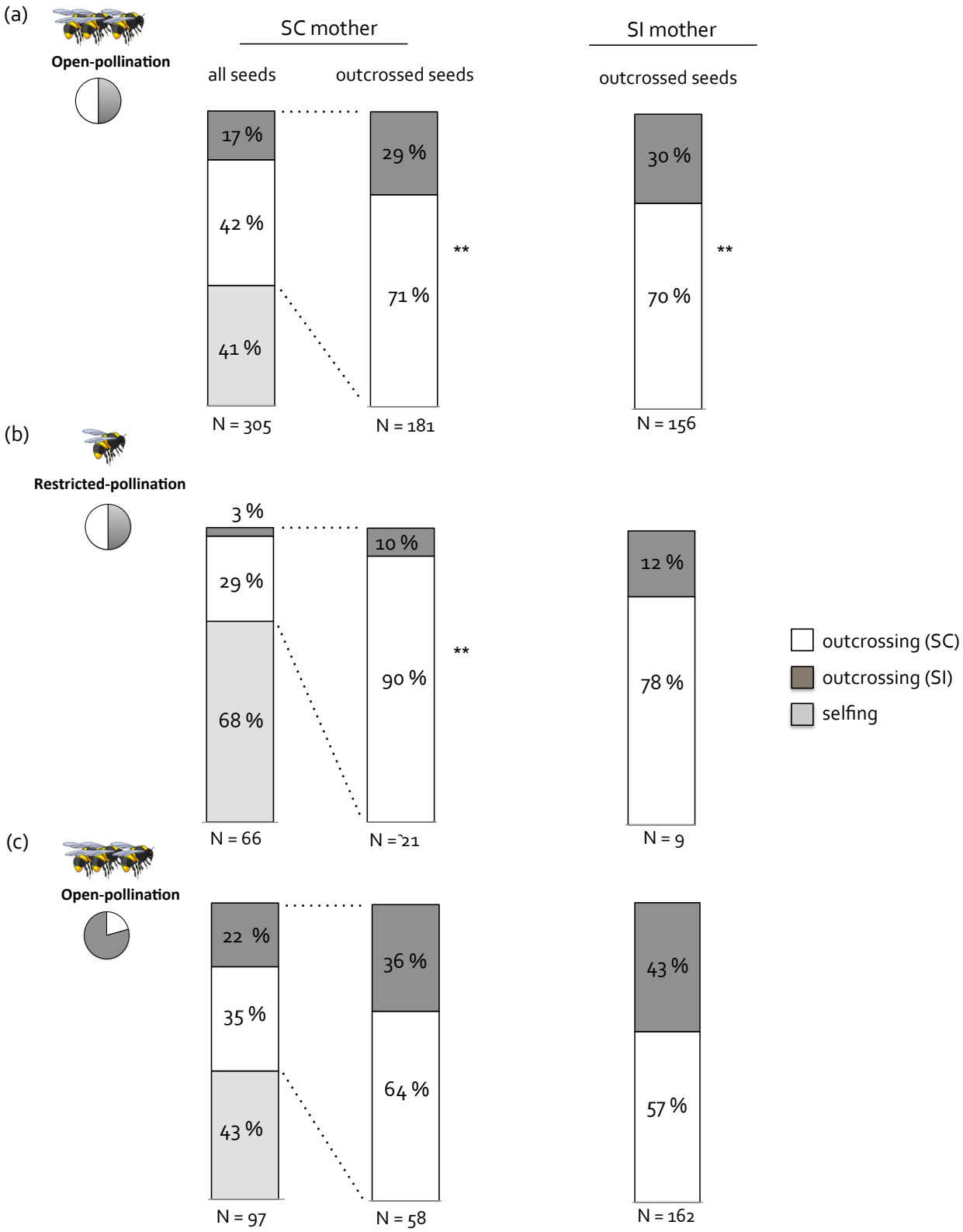

(a)
Open-pollination
SC mother
SI mother
all seeds
outcrossed seeds
outcrossed seeds
17 %
42 %
41 %
29 %
71 %
30 %
70 %
**
**
N = 305
N = 181
N = 156
(b)
Restricted-pollination
3 %
29 %
68 %
10 %
90 %
12 %
78 %
**
N = 66
N = 21
N = 9
(c)
Open-pollination
22 %
35 %
43 %
36 %
64 %
43 %
57 %
N = 97
N = 58
N = 162
outcrossing (SC)
outcrossing (SI)
selfing

**Figure 4. Selfing rates estimated for self-compatible (SC) plants of *Linaria cavanillesii*, after one generation of selection: (a) in the no-, restricted- or open-pollination treatments for experiment 1; and (b) in the open-pollination treatment for the experiment 2.** Initial proportions of SC and SI plants are represented by pie charts. Selfing rates were estimated using microsatellites and the program Colony (Jones and Wang 2010). Error bars are standard errors of the mean over two replicates for each respective treatment.

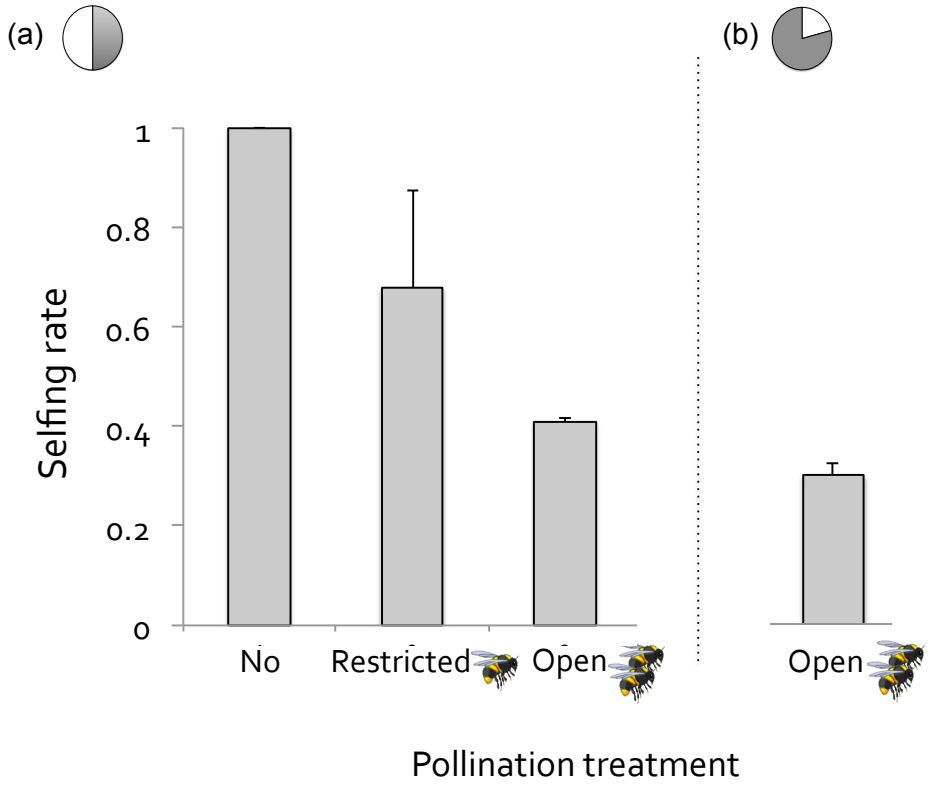
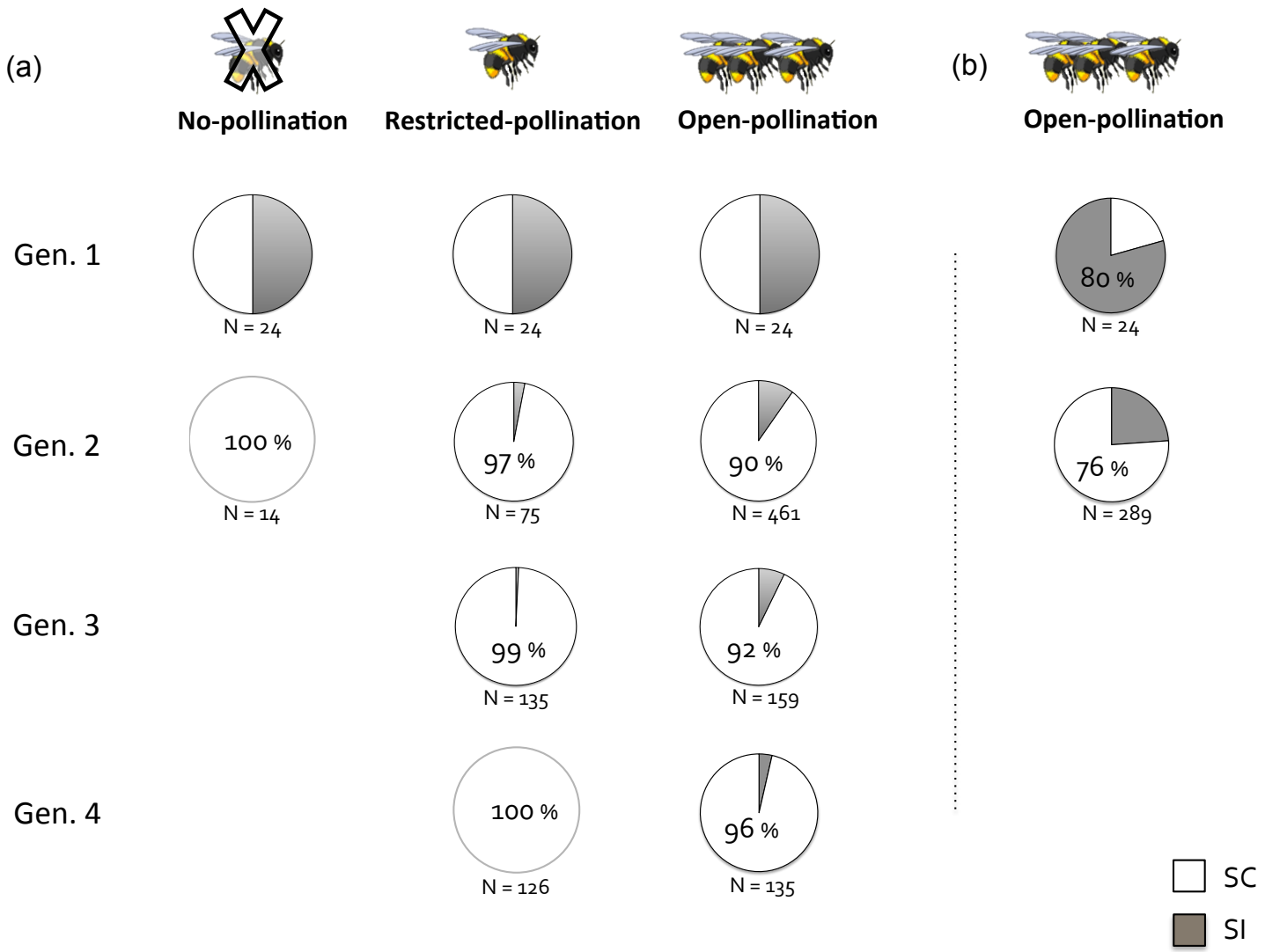


**Figure 5.** **Evolution of the proportion of self-compatible (SC) and self-incompatible (SI) individuals over three generations of selection in experiment 1 for: (a) the no-pollination, restricted-pollination or open-pollination treatments; and in experiment 2 (b) after one generation of selection in the open-pollination treatment.**



# SUPPLEMENTARY INFORMATION

**Table S1. Summary of the number of mother plants, the mean number of fruits per plant (fruits/plant), and the mean number of seeds per fruit (seeds/fruits) used to conduct paternity analysis in the selection experiment involving one self-compatible (SC) population and two self-incompatible (SI) populations of *Linaria cavanillesii*.**

| Pollination treatment | Compatibility phenotype | Mother plants | Fruits/plant | Seeds/fruit |
|---|---|---|---|---|
| Restricted 1 | SC | 1 | 3 | 12 |
| Restricted 2 | SC | 5 | 2 | 3 |
| | SI | 2 | 1 | 3 |
| Open 1 | SC | 8 | 2 | 9 |
| | SI | 8 | 2 | 6 |
| Open 2 | SC | 7 | 4 | 18 |
| | SI | 9 | 3 | 14 |
| No (control) | SC | 4 | 1 | 4 |

**Figure S1. Geographic location of the three populations of *Linaria cavanillesii* sampled in Spain, with their respective compatibility phenotypes as revealed by controlled crosses (SC: self-compatible population, SI: self-incompatible population).**

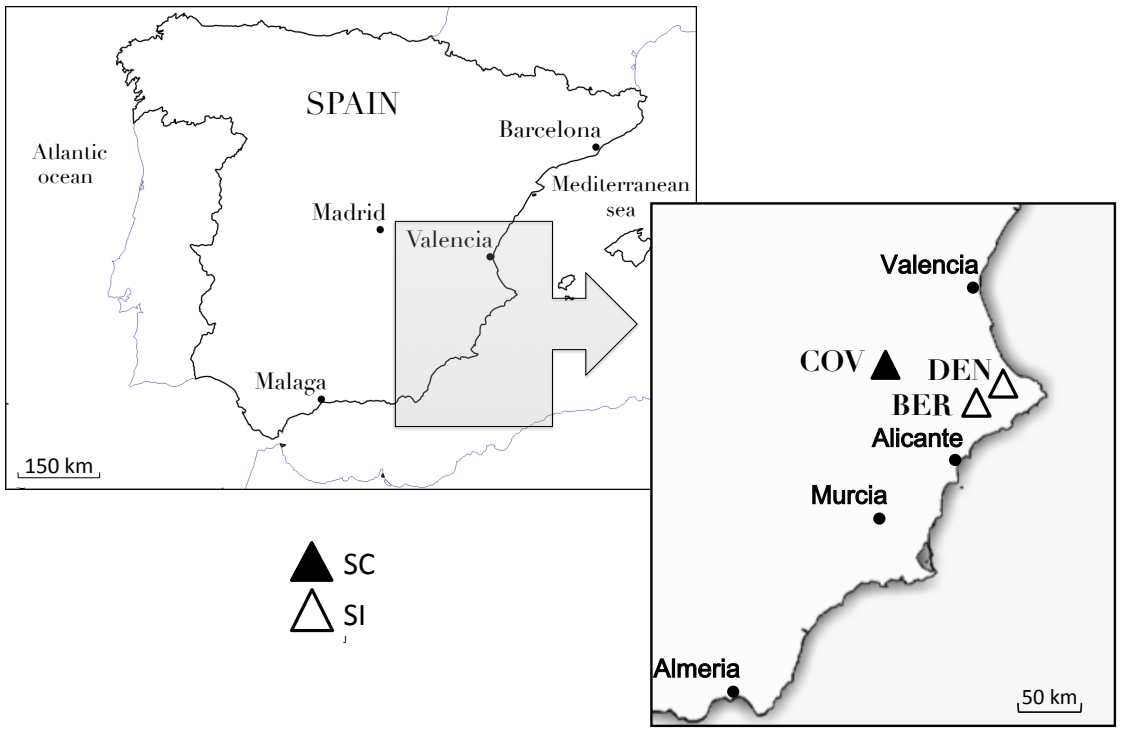


**Figure S2. Comparisons of flower production by self-compatible (SC) and self-incompatible (SI) plants after seven weeks of selection under (a) the open-pollination, restricted-pollination, and no-pollination treatments for plants in generation 1 of experiment 1; and (b) the open-pollination treatment for experiment 2.** The initial proportions of SC and SI plants are represented by pie charts. Fruit set was calculated as the mean fruit/flower ratio over all plants. In the boxplots, the middle lines represent medians, boxes represent the first and third quartiles and lower and upper bars represent the minimum and the maximum values. Red diamonds represent means. n.s indicates non significant differences between compatibility phenotypes.

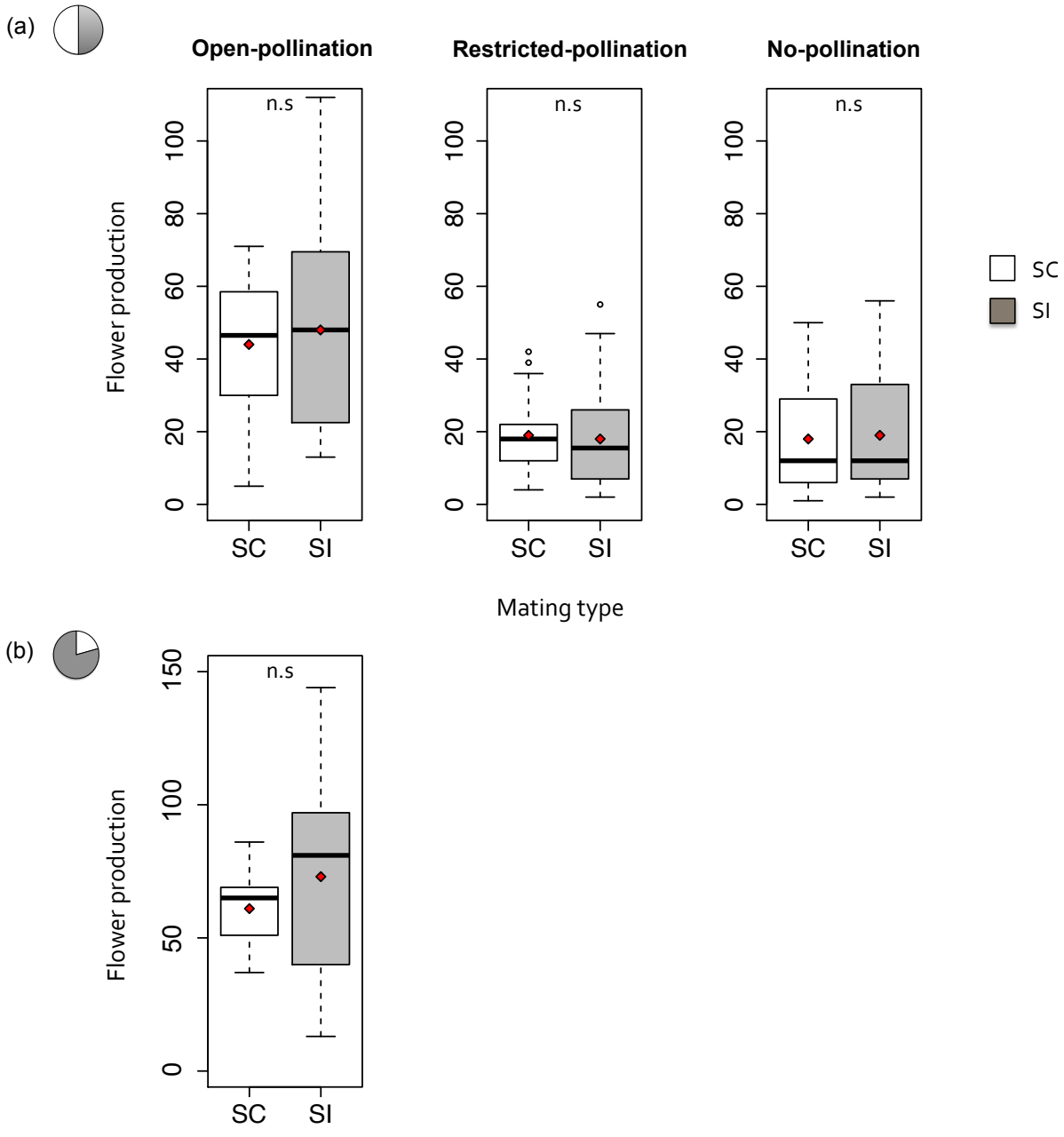


**Figure S3. Comparison of average seed production per fruit by self-compatible (SC) and self-incompatible (SI) plants under the open-, restricted-, and no-pollination treatments for plants in generation 1 of experiment 1**. Seeds were counted on every fruit produced over the course of two weeks. In the boxplots, the middle lines represent medians, boxes represent the first and third quartiles and lower and upper bars represent the minimum and the maximum values. Red diamonds represent means. n.s indicates non significant differences between compatibility phenotypes (note the absence of a test for plots under the no-pollination treatment, because there were no fruits produced by SI plants in the total absence of pollinators).

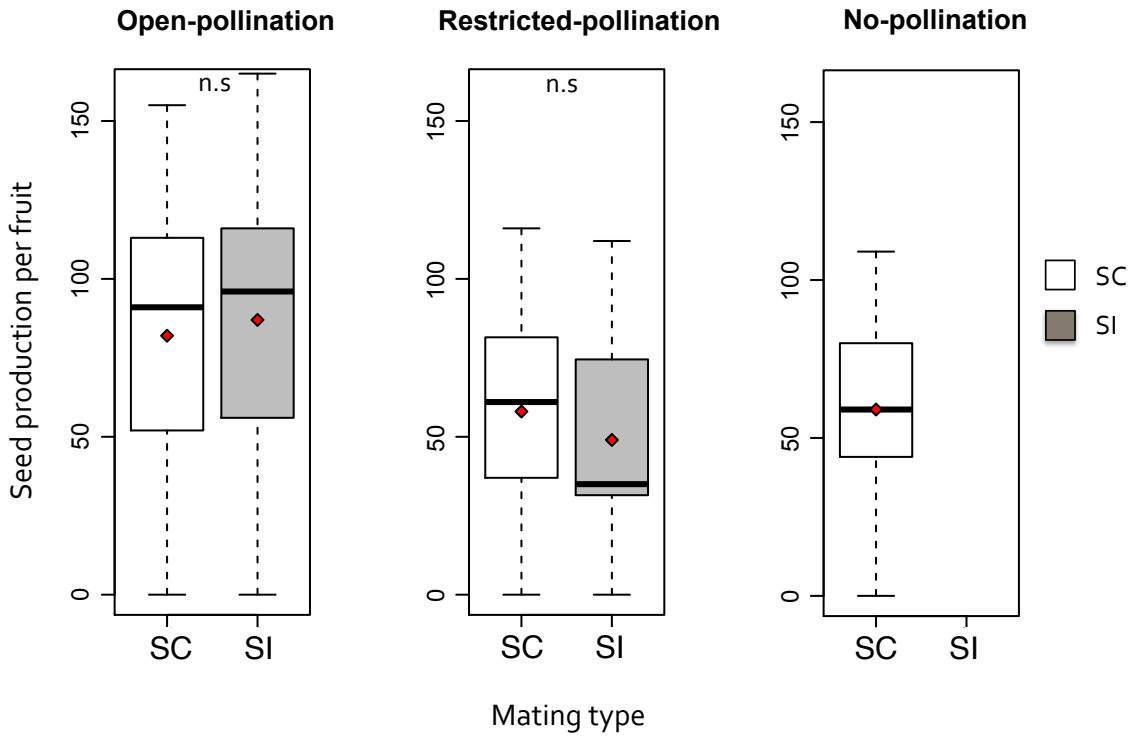


**Figure S4. Comparison of total seed production per fruit by self-compatible (SC) and self-incompatible (SI) plants under the open-, restricted-, and no-pollination treatments for plants in generation 1 of experiment 1.** Seeds were counted on every fruit produced over the course of two weeks. In the boxplots, the middle lines represent medians, boxes represent the first and third quartiles, lower and upper bars represent the minimum and the maximum values, and points represent outliers (i.e., points beyond 1.5 standard deviations)**.** Red diamonds represent means. * indicates significant differences between compatibility phenotypes (* : $P < 0.05$; ***: $P < 0.001$), and n.s non significant differences.

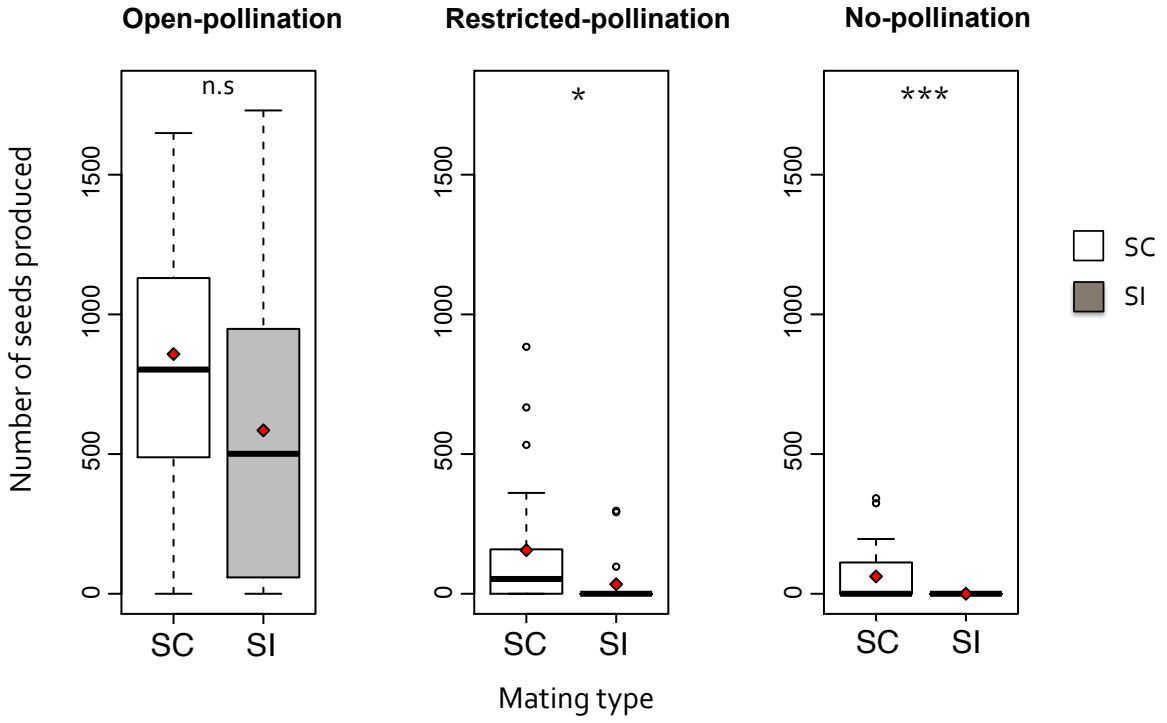
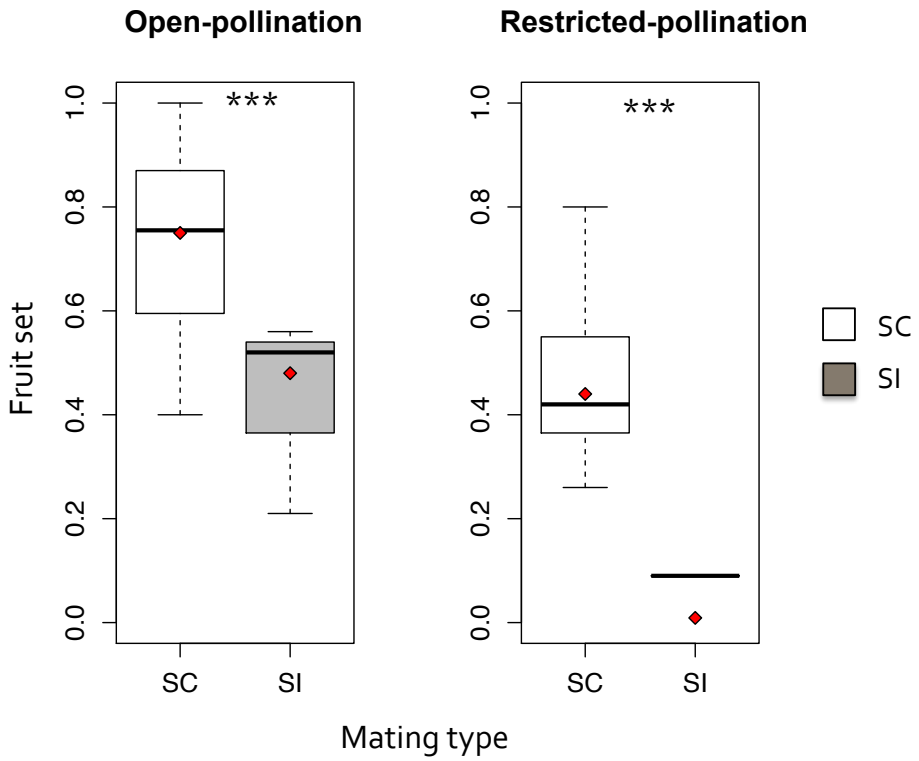


**Figure S5. Comparisons of fruit set by self-compatible (SC) and self-incompatible (SI) plants after five weeks of selection under the open- and restricted-pollination treatments for plants in generation 2 of experiment 1.** Fruit set was calculated as the proportion of fruits produced per flower by each plant. In the boxplots, the middle lines represent medians, boxes represent the first and third quartiles and lower and upper bars represent the minimum and the maximum values. Red diamonds represent means. * indicates significant differences between compatibility phenotypes (***: $P < 0.001$).



# - CHAPTER V -

## Pollen competitive ability promotes the invasion of self-compatibility in mixed populations of a perennial plant

M. Voillemot, S. Testa and J. R. Pannell

*(In preparation)*

# ABSTRACT

Evolution toward selfing is one of the most frequent transitions to have occurred in flowering plants, and its causes, mechanisms and consequences continue to pose puzzles. For instance, it is still not clear under what conditions newly self-compatible (SC) plants can invade self-incompatible (SI) populations of origin and if this is the case, what would be the speed of diffusion of SC. Additionally, because they do not need pollinators to mate, production of pollen is expected to be reduced in SC plants, which can reduce outcrossing opportunities. However and surprisingly, it has been found in a previous experiment that SC could spread rapidly in mixed populations, despite equal flower and pollen production compared to SI plants. In this study, we thus hypothesized that pollen competition could play a role helping this early spread by a higher siring success of SC plants. In order to test this hypothesis, we designed an experiment using the Spanish toadflax *Linaria cavanillesii*, from which approximately ten mother plants coming from both SC and SI populations were manually pollinated with different pollen mix. Paternity analyses revealed a significantly higher siring success of SC plants when competing with SI outcross pollen on SI plants, which can in part explain the rapid spread of SC in mixed populations.

**Keywords**: *Linaria cavanillesii*, mating systems, mixed mating, paternity, pollen competition, pollination, self-incompatibility, siring success

# INTRODUCTION

Many hermaphrodite flowering plants avoid self-fertilization via self-incompatibility (SI) (reviewed in Takayama and Isogai 2005). SI occurs in many families and orders (Franklin-Tong 2008) and in approximately 40% of all angiosperm species (Igic and Kohn 2006). Despite its likely benefits in preventing self-fertilization and thus avoiding inbreeding depression (Charlesworth and Charlesworth 1987), a frequent transition in angiosperms is the breakdown of SI mechanisms, leading a species/population to self-compatibility (SC) (Igic et al. 2008).

The breakdown of SI and the evolution toward increased selfing is expected to lead to altered pollen and ovule production (Ornduff 1969; Sicard and Lenhard 2011). Indeed, when plants shift to SC and increased selfing, they no longer need pollinators to ensure reproduction, and are expected to favour allocation of resources to ovule and seed production and away from investment in large floral displays, pollen, and nectar (Charlesworth and Charlesworth 1981). Although this 'selfing syndrome' may increase female reproductive success, it should also reduce outcrossing siring success (e.g. Fishman 2000). Indeed, reduced siring success through pollen discounting (the loss of pollen that might outcross due to the self-fertilization) has been shown to be a sufficient force maintaining outcrossing, despite low inbreeding depression (Holsinger 1991; Harder and Wilson 1998; Porcher and Lande 2005). Similarly, because flower size is expected to decline over time with selfing (Ornduff 1969), the resulting shorter styles can lead to reduced opportunities for pollen competition. Pollen competition has been shown to affect plant fitness, e.g. by increasing fruit set (Paschke et al. 2002) or offspring quality (Armbruster and Rogers 2004; Lankinen and Armbruster 2007; Pannell and Labouche 2013; Pélabon et al. 2015).

The pollen grains of selfing versus outcrossing plants are expected to experience divergent selective pressures that might affect their respective competitive abilities (reviewed in Mazer et al. 2010). First, we expect stronger selection on pollen competitive ability in outcrossers, mainly because flowers from outcrossers are usually larger and more attractive to pollinators (Goodwillie and Ness 2005), with

more diverse pollen samples competing to grow down longer styles. Second, pollen grains produced by outcrossers may end up on the stigmas of a wider diversity of maternal genotypes. Moreover, because male and female traits are not always under the same selection, antagonistic selection can lead to sexual conflict between alleles at the same locus. For instance, pollen grains may benefit from a fast tube growth to pollinate a maximum number of ovule, whereas female reproductive success would benefit by promoting competition among a large number of pollen grains per available ovule, e.g., by slowing tube growth down a longer style that remains receptive longer. However, such antagonistic selection should be reduced in selfers because of relaxed selection on male functions in selfing taxa (Mazer et al. 2010) where both male and female components of fitness apply to the same individual (Kerwin and Smith-Huerta 2000). One might thus expect evolution of pollen and style traits in a type of arms race in outcrossers, promoting strong pollen-tube growth but also strong resistance in styles, whereas, in selfing populations, this evolutionary dynamic would be absent. If so, pollen from individuals in an outcrossing population should outcompete that from individuals in a selfing lineage (reviewed in Brandvain and Haig 2005; see also Austerlitz et al. 2012; Rebernig et al. 2015), a prediction known as the WISO (weak inbreeder/strong outcrosser) hypothesis (Brandvain and Haig 2005). In a review of the evidence, Brandvain and Haig (2005) found strong overall support for the WISO hypothesis, albeit with a number of clear exceptions.

The WISO hypothesis has been formulated in terms of differences in pollen performance between outcrossing and selfing populations in general, but it seems less clear to know what to expect when looking at pollen from mixed-mating populations with intermediate selfing rates. For instance, what should we expect while comparing competition between pollen from an obligate outcrossing population and pollen from a mixed-mating population in which individuals still compete for outcrossing opportunities? Whereas one might simply expect a diminished difference in performance between pollen sources in this case, Lankinen and Armbruster (2007) predicted selection for competitive pollen in mixed-mating populations that carry substantial genetic load, and thus where selfing leads to inbreeding depression. Either way, the nature and strength of selection acting on outcrossers versus selfers is likely to depend on the time of selection. But how rapidly should the

effects predicted by the WISO hypothesis become evident? In their original paper, Lewis and Crowe (1958) analysed the result of crosses from SI and closely related SC plants. They noticed exception to the WISO rules in only four cases, and they all involved species that had recently lost SI.

Here, we test the WISO hypothesis using crosses between individuals from SI populations of the perennial herb *Linaria cavanillesii* and those from a lineage that has only recently lost SI and underwent a transition to mixed mating (Chapter I). While the SI populations of the species express high levels of inbreeding depression upon selfing (made possible by a degree of 'leakiness' of SI), inbreeding depression has been purged from the SC population (Chapter II). Given this absence of inbreeding depression, we would expect selection to favor evolution of a 'selfing syndrome', i.e., the production of smaller, less rewarding flowers with lower pollen/ovule ratios (Ornduff 1969; Sicard and Lenhard 2011). However, the SC population of *L. cavanillesii* continues to show floral traits in common with its SI counterparts, i.e., large inflorescences and flowers that produce similar quantities of nectar, and similar pollen/ovule ratios; and individuals that are frequently visited by pollinators display intermediate to high outcrossing rates under natural conditions (Chapter I). Modeling using approximate Bayesian computation (ABC) has inferred a transition from obligate outcrossing to mixed mating in *L. cavanillesii* more recently than about 3,000 generations (Chapter III), suggesting that while there has evidently been sufficient time for inbreeding depression to be purged from the SC population, the transition to SC has perhaps been too recent for the evolution of a selfing syndrome.

Against this background, we ask here whether modified selection implied by the WISO hypothesis has brought about differences in the siring ability between pollen from the mixed-mating SC versus the SI populations. We addressed this question experimentally by placing equal quantities of pollen from SI and SC individuals on stigmas of differing genotypes and using molecular markers to assess the paternity of the seeds sired. Our study is also motivated by results from a recent experiment in which we found that SC individuals were substantially better sires than SI individuals in experimental populations exposed to pollinators (Chapter IV). It is possible that the results from that experiment were influenced by differences in pollinator behavior, or by incompatibilities between SI individuals

that might have compromised their siring success – although both these possibilities were unlikely (Chapter IV). Our current study tests the hypothesis that the greater siring success of SC individuals in the experimental mating arrays can be attributed to greater competitive ability of their pollen, in contrast with what is predicted by the WISO hypothesis.

# MATERIALS AND METHODS

*Study species*

*Linaria cavanillesii* (Plantaginaceae) is a long-lived perennial herb endemic to southeastern Spain. The species is largely SI (Chapter I), but a SC population is known from the north of its range (Carrió et al. 2013; Chapter I), and some populations are mainly SI but also comprise individuals with leaky SI. Each spring, plants sprout from a rootstock and grow numerous shoots, with leafy inflorescences. Flowers are large and attractive and reward a variety of bees and bumblebees with copious nectar presented in a nectar spur. With the flower, stigmas are immediately surrounding and touched by four anthers, so that SC leads to autonomous selfing in the absence of insect visitors. However, insect visits bring about some outcrossing even in the SC population, which thus have a mixed mating system (s = 0.59; Chapter I). After successful pollination, flowers transform into fruit capsules, which release seeds passively when mature. We conducted this study using plants from six populations with well-characterised levels of compatibility (SI: BER and DEN; SC: COV; leaky SI: RUB, BUI and ZAR; Chapter I).

*Pollen competition experiment*

To study the effect of pollen competition on siring success between plants of different compatibility types, we selected 16 plants as pollen donors and 23 plants as dams (see Supplementary Table 1 for details). All plants were grown in the glasshouse of the University of Lausanne, Switzerland, from seeds collected in the field. For each dam, we pollinated four flowers with the following pollen

mixtures: (1) self + SC outcross, (2) self + SI outcross, (3) SC outcross + SI outcross, or (4) control with a single pollen donor.

Before pollination, we emasculated closed flowers by removing anthers with sterilized sharp tweezers. From this moment on, we covered every emasculated flower with a small nylon bag to avoid pollen contamination. Three days later, when flowers were fully open, we applied pollen accordingly to the treatments described above. For mixed pollen treatments, we collected two anthers from each potential father, broke them with sterilized tweezers and mixed them thoroughly on a microscope slide. For the control treatment of pollination with a single pollen donor, we used the same protocol but collected pollen from four mature anthers of the same plant. The mix of pollen was applied until the stigma was fully covered by pollen. Approximately 20 days after pollination, we applied rubber glue to the apex of fruit capsules to avoid the dispersion of seeds, and collected seeds ten days later when they were fully mature. We germinated approximately 30 seeds per mother and collect leaves from the progeny for genotyping and paternity assignment.

*Paternity analyses*

For paternity analysis, we extracted DNA from about 28 (min: 6, max: 34) progeny per treatment and dam combination, as well as from their mothers, using the DNeasy 96 Plant kit (Qiagen). We amplified six polymorphic microsatellite primers by PCR (Biometra thermocycler; Göttingen, Germany) using the following reagents: 1xPCR mix: 2 ng/µL template DNA, 10x PCR Buffer, 25 mM $MgCl_2$, 5x Q-solution, 2.5 mM dNTP, 0.2 µM of each primer and 0.5 U/µL of Taq DNA polymerase (HotStarTaq®, Qiagen). The thermocycling conditions followed the protocol described in Chapter I and involved 15 min at 95° followed by 32 cycles for 30 sec at the annealing temperature, 30 sec at 72 °C and 30 sec at 95 °C, followed by one cycle for 1 min at the annealing temperature, and a final extension for 30 min at 72 °C, PCR products were then sequenced in an ABI3100 (Applied Biosystems). We used the program Genemapper® to analyze microsatellite data, and the program Colony® to infer paternity using maximum likelihood estimates. To investigate differences in siring

success among pollen competition treatments, we averaged proportion of mating success for each dams in each respective treatments, and compared means against random mating (mean = 0.5) using one-sample Students' *t*-test. All statistics were conducted in R (version 3.1.2, R Core Team 2015).

# RESULTS

Compared to SI pollen, pollen from SC plants was more successful at siring outcrossed progeny on SI dams (SC pollen sired 70% of the seeds produced), and this was significantly different from random mating ($t_{11}$ = 2.66, $P$ = 0.02; Figure 1a). By contrast, there was no average difference in the siring success between compatibility morphs on SC dams (mean: 0.55 ± 0.05 SEM; $t_9$ = 0.93, $P$ = 0.38, Figure 1b). Nor did we find any significant difference in siring success between self and SI outcross pollen ($t_9$ = -0.13, $P$ = 0.90; Figure 1c). Similarly, although there was an overall tendency for self-pollen to have been more successful than outcross pollen from other SC plants in siring seeds (self pollen sired 64% of the seeds produced), variation between fruits was substantial, and the difference in siring success between self and SC outcross pollen was not significant ($t_7$ = 1.62, df = 7, $P$ = 0.15; Figure 1d).

# DISCUSSION

In this study, we assessed the relative competitive abilities of pollen from self-compatible (SC) versus self-incompatible (SI) individuals of *Linaria cavanillesii*, with a view to testing the WISO hypothesis of Brandvain and Haig (2005) that predicts more competitive pollen from SI lineages, as well as to account for observations of greater siring ability of pollen from SC individuals observed in experimental populations, in contrast to expectations of the WISO hypothesis. We found that SC pollen sired significantly more progeny on SI plants than pollen from other SI individuals (on average, 67 % of the progeny), a result that runs counter to the WISO hypothesis and helps to explain the results of our previous experiment. It would thus seem that SC pollen is indeed more competitive than SI pollen, irrespective of which populations were used in the crosses. Below, we first attempt to

interpret the patterns of competitive ability of pollen in *L. cavanillesii* in the context of the WISO hypothesis. We then discuss the implications our results have for understanding the mating system of this species, and the loss of SI and mixed mating in general.

*Variation in siring ability among pollen sources and maternal backgrounds*

Given the competitive superiority of pollen from SC individuals of *L. cavanillesii* in SI styles, we might have expected a similar result when SC pollen competed against SI pollen in SC styles. However, we found no difference between the two pollen sources in this context. It is difficult to explain why pollen from SC plants should compete better with that from SI pollen only in styles of SI individuals. However, scrutiny of our data reveals that pollen from the ZAR population, as well as pollen from two individuals from the BUI population, were always better competitors in our crosses, irrespectively of the other competitive pollen or the mother plant. If we exclude these crosses from our analysis, a significantly greater proportion (65%) of the remaining seeds were sired by SC plants, i.e., similar to the levels observed in the previous field experiment (Chapter IV). Of course, we had no *a priori* reason to exclude any of the crosses, but, as we discuss next, it is possible that the ZAR population, specifically, may have been subject to a different intensity of selection than the other populations.

The ZAR population of *L. cavanillesii* stands out among those we have sampled by being the only SI population not to show any evidence of pollen-limited seed set; in contrast to all other SI populations studied where seed-production was pollen-limited in the years we studied them (Chapter I). Consistent with this difference, the ZAR population was also much larger than all the other populations sampled, and enjoyed particularly high pollinator visitation rates (Chapter I). It is thus plausible that a high competitive ability of pollen tubes has been particularly strongly selected in this population, so that its pollen would be more competitive in a race to fertilize stigmas with pollen from anywhere else, including the SC population. For instance in the SI plant *Cochlearia bavarica,* hand-pollination crosses revealed that increasing the number of pollen donor had a positive effect on reproductive

success, but this was not due to pollen load size but rather to the increased pollen diversity among pollen donors (Paschke et al. 2002).

Taken at face value, the higher siring success of SC plants, at least in SI styles, is somewhat surprising given the expectation of the WISO hypothesis, which predicts greater competitive of pollen from outcrossers (Brandvain and Haig 2005; Austerlitz et al. 2012). However, recall that SC individuals of *L. cavanillesii* are still highly attractive to pollinators and continue to outcross a substantial number of their seeds (Chapter I). It is also most likely very recently derived from SI populations (Chapter III), and might thus be expected to show pattern of pollen competition displayed by outcrossers (Mazer et al. 2010). We speculate that, in mixed-mating populations such as the SC population of *L. cavanillesii*, selection for a high competitive ability of pollen might actually be higher than in a pollen-limited outcrossing population, because pollen deposited on stigmas of other individuals will always have to compete with substantial numbers of self pollen grains. If so, the explanation demands a more nuanced WISO hypothesis that accounts for levels of pollen limitation under mixed mating.

We found no differences in siring success between self-pollen vs. SI outcross pollen, but a higher siring success of self-pollen vs. SC outcross pollen. This result is puzzling. Self-pollen has previously been found to perform better than outcross pollen on its own style, perhaps because of reduced genetic conflict between the pollen and its own maternal genotype (Kerwin and Smith-Huerta 2000) and reduced opportunity for sexually antagonistic selection under selfing (Tazzyman and Abbott 2015). However, in their study, Kerwin and Smith-Huerta (2000) also found that the same pollen was a worse competitor when in competition on other maternal genotype, which was not the case in our study here. Again, we suggest that a history of outcrossing (in the context of mixed mating) might have selected for competitive pollen, and selfing events might have reduced maternal-paternal conflicts.

*Implications for explaining mixed mating and the spread of SC in* L. cavanillesii

By comparing the competitive abilities of pollen between SC and SI populations in *L. cavanillesii*, our study has contributed new data that further challenge the generality of the WISO hypothesis and perhaps demand a more nuanced version of it, but our results also contribute to understanding the mating-variation in *L. cavanillesii*. Two aspects are worth highlighting. First, the fact that pollen from SC individuals does appear to have a competitive advantage over that from SI individuals, at least in certain crosses, helps to explain why, in experimental populations of both SI and SC individuals exposed to pollinators, SC individuals were more frequent sires of progeny than SI sires. It is possible that pollen transferred between some SI individuals was rejected by the incompatibility reaction, and that SC plants attracted more pollinators that SI individuals, as suggested in the Introduction. However, our results here suggest that an important component of the siring advantage of SC individuals can be attributed to more competitive pollen-tube growth. We do not know what the mechanisms behind our result might be, e.g., whether they are the result of an intrinsic difference in the strength of pollen-tube growth, or are influenced by maternal selection of pollen tubes (Skogsmyr and Lankinen 2002; Pannell and Labouche 2013; Lankinen and Karlsoon Green 2015).

Second, our results throw new light on evolution of traits affecting the mating system in *L. cavanillesii* in the wake of its transition to SC in part of its range. As in many species that present a mixed-mating system (Goodwillie et al. 2005), the maintenance of intermediate selfing rates and an outcrossing floral syndrome in *L. cavanillesii* has been puzzling. The currently most plausible scenario to explain the patterns observed invokes the idea that SC evolved sufficiently long ago for inbreeding depression to have been purged, perhaps during periods of severe mate and/or pollinator limitation, but too recently for the evolution of reduced allocation to pollinator attraction and reward (Chapter I and III). Into this scenario we can now incorporate the evolution of traits that affect variation in pollen competitiveness in the derived SC lineage. Although our results largely run counter to those predicted by the WISO hypothesis, they do indicate that populations of *L. cavanillesii* differ not only in their incompatibility status and the levels of inbreeding depression expressed upon selfing, but also in the

behaviour of their male gametophytes. Our results thus suggest that pollen competitiveness can diverge among populations at a rate that might be equivalent to that of the purging of inbreeding depression, but certainly more rapidly than floral morphological and allocation traits. The extent to which the patterns we have observed might be affected by mixtures of pollen from more than two potential sires (e.g., Travers and Holtsford 2000; Shaner and Marshall 2007; Madjidian et al. 2012 but see Marshall et al. 2007) or deposited at different times (e.g. Lankinen and Strandh 2016; Sorin et al. 2016) remain to be seen.

## ACKOWLEDGMENTS

We wish to thank Ana Paula Machado for help with microsatellites analysis and funding from the University of Lausanne to JRP in support of a studentship to MV.

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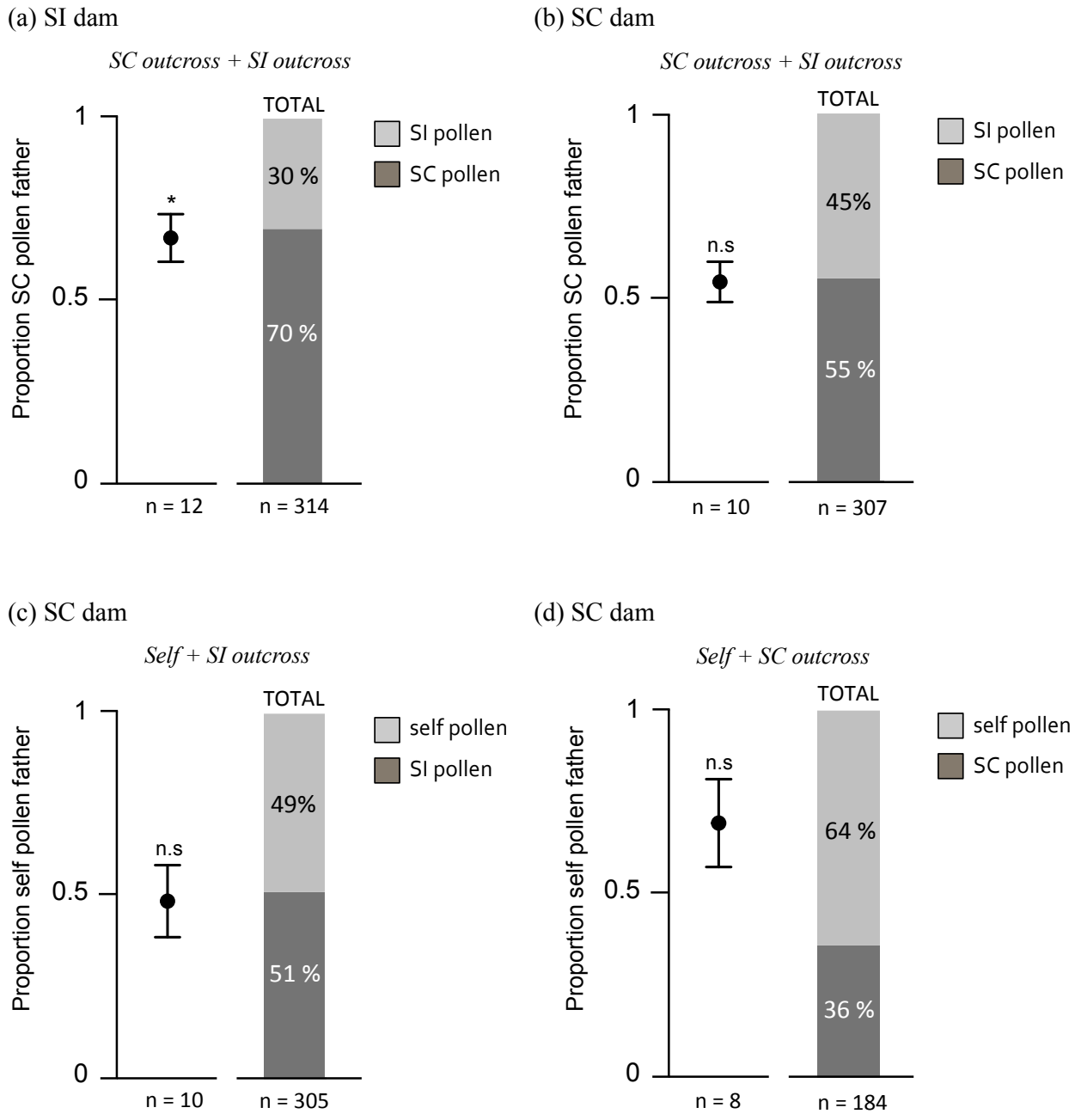
**Figure 1. Comparison of paternity for different dams (SI: self-incompatible; SC: self-compatible) and pollen treatments**: (a) and (b) SC outcross + SI outcross; (c) self + SI outcross or (d) self + SC outcross. The two potential pollen sires are indicated in dark and light grey for each respective treatment. Bars on the left represent mean +/- SD with significant differences from a Student *t*-test indicated. Bars on the right indicate the total siring proportion of each potential father.



**Supplementary Table 1. Summary of mother and pollen donor plants used in the pollen competition experiment with *Linaria cavanillesii*.** Plants come from six populations, either self-compatible (SC), self-incompatible (SI) or leaky self-incompatible containing SC plants (indicated with a *).

| Mother mating | Mother ID | SC pollen donor | SI pollen donor |
|---|---|---|---|
| SC | COV_111.2 | COV_393.9 | BER_303.2 |
| | COV_119.1 | BUI_320.1* | BUI_312.3 |
| | COV_384.1 | COV_111.2 | NA |
| | COV_389.6 | COV_399.1 | BUI_314.1 |
| | COV_390.X | RUB_163.9* | BER_310.4 |
| | COV_392.4 | COV_174.3 | ZAR_61.3 |
| | COV_396.3 | COV_392.4 | BER_90.1 |
| | COV_399.1 | COV_398.3 | DEN_8.8 |
| | DEN_130.2* | COV_389.6 | BUI_313.4 |
| | ZAR_60.4* | RUB_165.111* | BER_90.1 |
| | ZAR_95.3* | RUB_163.9* | DEN_120.2 |
| SI | BER_303.2 | COV_119.1 | ZAR_202.2 |
| | BER_305.4 | RUB_165.111* | DEN_16.1 |
| | BER_309.6 | COV_396.3 | BUI_312.3 |
| | BER_310.4 | COV_392.4 | BUI_40.111 |
| | BER_96.111 | COV_393.9 | ZAR_61.3 |
| | BUI_314.1 | COV_387 | BER_303.2 |
| | DEN_120.2 | COV_171.2 | BER_90.1 |
| | RUB_138.2 | COV_396.3 | BUI_314.1 |
| | DEN_8.8 | COV_111.2 | BER_305.4 |
| | RUB_165.1 | BUI_320.1* | DEN_120.2 |
| | ZAR_202.2 | COV_174.3 | BER_310.4 |
| | ZAR_61.3 | COV_389.6 | BER_309.6 |

# - GENERAL DISCUSSION -

# *GENERAL DISCUSSION*

In this thesis, I have attempted to understand the evolutionary forces that maintain contrasting reproductive strategies in flowering plants, notably self-incompatibility (SI) vs. self-compatibility (SC), and selfing vs. outcrossing. Species presenting a recent or on-going transition towards SC are particularly useful for investigating in detail the forces that can prevent a shift to SC and/or maintain SI. With the observation of among-population variation in SI and the mating system, as well as the estimation of the divergence time between SC and SI populations, *Linaria cavanillesii* joins the few studied species presenting within-species SI vs. SC polymorphism and a probable recent breakdown of SI (e.g. Lloyd 1965; Raven 1969; Busch 2005; Mable et al. 2005; Dart et al. 2012).

Given the broad diversity of mating systems that can be observed in nature, it is hard to imagine that we can classify species as simply belonging to the SI or SC group. However, some common conventions, mainly because of simplicity, have considered that populations or plants showing outcrossing rate above 0.8 would be considered as SI (Schemske and Lande 1985). We do not intend to classify populations as being in a stable state, because we can always ask whether a mating system is evolutionary stable or not. Ideally, mating systems should be measured on a maximum number of individuals across the species distribution, and similar individuals should be measured several times. However, this in practice is difficult to achieve. An index of SI was first introduced by Lloyd (1965), and represents the relative measure of reproductive success following hand self- and cross-pollinations. In order to question the classical view of bimodality of SC vs. SI, Raduski *et al.* (2012) recently used this ISI index to assess variation in SI from more than 1200 angiosperm taxa, and results showed that the distribution is indeed bimodal and positively associated with outcrossing rates. After growing many *L cavanillesii* plants in the greenhouse, and based on an among-population variation that was quite clear, we thus distinguished broadly SI, leaky SI and mixed-mating SC populations.

In this brief general discussion, I first outline the main conclusions about the transition towards selfing in *L. cavanillesii*, and how these might contribute to a broader understanding of mating-system evolution in plants. Finally, in a brief section I turn to several of the puzzles that have either not been answered by my research, or that are posed by the results obtained. Here, I also mention the sorts of studies that might still profitably be conducted on this study system to address general questions.

## Main conclusions

### *The evolution of SC in* L. cavanillesii

Through its natural range, most populations of *L. cavanillesii* are considered SI, as expected for a long-lived perennial species (Munoz et al. 2016). Even if they do not seem to suffer from limitation in compatible mates, as revealed by the apparent diversity of S-alleles present in SI populations (Appendix 2), almost all SI populations show patterns of reproductive failure due to pollen limitation. SC can confer the benefit of reproductive assurance despite an absence of pollen vectors, and we may thus expect this benefit to favor mutations conferring SC in these populations. However, despite some evidence for leaky expression of SI in some of its populations, a shift to SC seems to be prevented by strong inbreeding depression in these. Inbreeding depression is one of the most important forces that can prevent shift to SC (Charlesworth and Charlesworth 1987), and, as in many other species (Escobar et al. 2011; Winn et al. 2011), here again it seems to be sufficient to prevent the spread of SC despite the benefit that reproductive assurance could bring.

In contrast to many species where selfing has evolved to limit the effect of pollen limitation, pollinators were found sufficient to ensure seed set in the one known SC population of *L. cavanillesii*, despite an ability to set fruit autonomously. Several aspects seem to indicate that the presence of pollinators and attractiveness of flowers may explain the maintenance of outcrossing ($s = 0.59$) in this population, as has been found in other species (Kalisz and Vogler 2003, 2004; Dart et al. 2012). For instance, while setting up our experimental mixed arrays under different pollinator densities, we found relatively similar outcrossing rates in the open-pollination array compared to natural populations. It has been previously suggested that an optimal mating system would be to reproduce by selfing and thus benefit from reproductive assurance during colonization process, whereas outcrossing may be favoured when pollinators become less limited (Pannell and Barrett 1998). I suggest that the mixed mating we observe in the SC population of *L. cavanillesii* is context-dependent, and that shifts towards increased selfing in the absence of pollinators or mates are likely to occur after a period of population establishment. However, the maintenance of outcrossing in *L. cavanillesii* is still puzzling considering the absence of inbreeding depression found for this population. Mixed-mating species has been shown to harbour similar levels of inbreeding depression than their outcrossing counterpart (Winn et al. 2011), and we can hypothesise that, in the absence of inbreeding depression, the SC population should shift to increased selfing over time. Again, because it is a long-lived perennial plant, we may expect temporal variation in selfing rates and thus a shift to increased selfing in the case of a deficit of pollinators in a longer term.

Several aspects seem to point to a scenario where the SC population of *L. cavanillesii* resulted from a strong bottleneck and/or founder event, followed by expansion of the newly colonized population with

purged inbreeding depression. First, the loss of SI following a strong bottleneck can lower the genetic diversity in the colonized population, increase the genetic load and accelerate purging (Kirkpatrick and Jarne 2000), which could eventually explain the absence of inbreeding depression found in the SC population. Secondly, the absence of a selfing syndrome is likely to be the result of a recent shift toward SC (Foxe et al. 2010; Busch et al. 2011; Carleial et al. 2016), as well as from little standing genetic variation after a bottleneck. Indeed, following the expected consequences of the breakdown of SI and increased selfing rates, the recently derived SC population of *L. cavanillesii* presents reduced genetic diversity compared to SI populations, as well as strong patterns of differentiation among populations, which was also confirmed by some levels of heterosis among distant populations. However, in a recent meta-analysis, Vos et al. (2014) compared 124 primrose species to investigate whether a selfing syndrome evolved in each species after an independent loss of SI. They found some evidence of evolution toward a selfing syndrome in some cases, but this was not universal, and the authors concluded that morphological changes do not necessarily evolve in response to increased selfing. The explanations were diverse and not mutually exclusive, with a transition to SC that can have resulted in an increase in genetic drift (and thus a reduced effective population size), a small amount of genetic variation, reduced directional selection on floral traits, as well as pleiotropic effects that may constrain the correlated evolution of floral traits. Many hypotheses can thus explain the absence of evolution toward a selfing syndrome after the loss of SI. In the case of *L. cavanillesii*, some of these hypotheses probably act in concert, resulting mainly from the recent divergence time following a population bottleneck.

*Mating system polymorphism*

Despite the mating-system continuum found in natural populations, it is relatively rare to find species presenting populations mixed with strictly SI or SC individuals. In contrast, it is common to find SC species with various degree of selfing rates (mixed mating, Goodwillie et al. 2005), or sister species with an ancestral SI species and a recently derived SC species (e.g. Tang et al. 2007; Guo et al. 2009; Foxe et al. 2010). When occurring together with SI plants in different pollinators environment, we found evidence for both an advantage of reproductive assurance and the automatic selection of the SC plants compared to SI plants, which resulted in a shift towards complete SC in a few generations only. Additionally, there was no evidence for pollen discounting in SC plants, a force that could be sufficient to prevent a shift to SC and increased selfing (Harder and Wilson 1998; Porcher and Lande 2005). It is thus puzzling that SC has not spread through the species range. We hypothesize that, in natural populations of *L. cavanillesii*, strict isolation is one of the key factors maintaining mating system polymorphism. However, the experiment started with an initial proportion of SC plants of 50% or 20%, in mixed arrays. It would then be really interesting to know 1) what would happen if only one SC plant was present at the beginning (such as would probably happen in a scenario of invasion in

natural populations), 2) how much the outcome can be influenced by the total amount of plants in the populations, and 3) what would happen in the following generations (for instance, how much inbreeding depression would start to buffer the advantage of transmission of SC plants and to know if the population would be stable over time). These questions are currently assessed using simulations and the help of a collaborator, and with these we hope to gain more insights about the spread of SC in SI populations.

Pollen discounting is an important force that can prevent a shift toward increased selfing and thus maintain mixed mating in SC species (Harder and Wilson 1998; Porcher and Lande 2005). However, added to the fact that SC plants produce the same amount of pollen as SI plants, we also did not find any evidence for pollen discounting in the SC population, since SC pollen grains were better sires than pollen from SI individuals, irrespective of their attractiveness to pollinators. Asymmetrical selection on SC vs. SI pollen has been observed previously (Mazer et al. 2010), but it is usually the SC pollen that is expected to show reduced competitiveness compared to outcrossed pollen, and it is still not clear here what drives the counterintuitive outcomes. Perhaps the fact that the transition to SC has been recent, combined with the maintenance of mixed mating and the strong isolations of populations would in part explain this (Mazer et al. 2010). However, in the controlled experiment, we deposited a mix of pollen on stigma simultaneously and in the same proportion. In natural conditions, we can expect this to vary, and it would thus be revealing to perform further experiments in which the pollen mix was varied.

*Conclusion*

Self-fertilization is often seen as an evolutionary "dead-end" (Escobar *et al.*, 2010; Goldberg *et al.*, 2010; Wright *et al.*, 2013; but see Igic and Busch, 2013), in part because SC evolves in marginal, or isolated environments, which experience extinction by short-term ecological variation and as a result of the consequences of genetic drift in the long term. In *L. cavanillesii*, all populations we studied are isolated, irrespectively of their mating system, and we found strong population structures among the SI populations, with a clear patter of genetic isolation by distance. Thus, due to restricted population size and isolation, it seems more likely that extinction could affect any part of the population range, irrespectively of the mating system. At the end of his thesis, Busch, (2005b) drew conclusions concerning the fragility of SC populations of the species *Leavenworthia alabamica*, in part because he observed the extinction of two out of the five SC studied populations during his study. As he mentioned, the transition towards SC in the genus *Leavenworthia* often resulted in a complete loss of genetic variation, which can make SC populations particularly vulnerable to environmental changes, and it thus seems that reproductive assurance and colonization of new disturbed habitat probably drives the frequent transitions towards increased selfing. In many aspects, *L. cavanillesii* populations

share patterns in common with *L. alabamica*. The one known completely SC population of *L. cavanillesii* probably resulted from a strong bottleneck as well, accounting for its lower genetic variation compared to its SI sister populations. Similarly, inbreeding depression seems to be one of the major forces maintaining outcrossing and SI in other populations. However, in contrast to *L. alabamica*, it does not seem to me that the known SC population is particularly prone to extinction. Instead, it is one of the largest populations, and plants seem not to suffer directly from the reduced genetic variation, because they still reproduce and survive as or better than SI plants. Even if we cannot predict how reduced genetic variation might impact the SC population in a longer term compared to SI populations (Morran et al. 2009), *L. cavanillesii* is a perennial plant species that seems likely to be able to tolerate poor conditions by resprouting in the following spring. For instance, during the 2014 reproductive season, populations experienced a severe drought, but this did not seem to affect plants as they flowered normally the following year. However, even with their apparent resistance to environmental stochasticity, I would suggest that SI populations are in fact more vulnerable than the SC one, mainly due to their mating system that is dependent on available mates and pollen vectors. Given the small populations sizes, *L. cavanillesii* may experience recurrent extinction/colonization processes, but the extreme specific ecological requirements of the species may affect the persistence of the species in the longer term. I would find particularly valuable to search in more detail for other SC populations in the small range of *L. cavanillesii*. If other SC populations occur, knowing whether they result from the same event or independent transitions would be particularly illuminating.

### *Unanswered questions and perspectives*

#### *Genetic mechanisms of SC evolution and transmission*

One aspect that was not investigated in this thesis is the genetic aspect of the breakdown of SI and transmission of SC in *L. cavanillesii*. I know from observations that SC is passed to the progeny whenever a cross involves an SC partner. However, I do not know in any detail the genetic mechanisms of transmission by SC individuals or the causes of the SI breakdown. For this, performing more controlled crosses as well as sequencing the S-locus in SC vs. SI populations would be particularly worthwhile, mainly because it can also help reconstructing the history of the loss of SI in this species (Vekemans et al. 2014). Additionally, while performing experiments on the campus of the University of Lausanne, I found some hybridization between *L. cavanillesii* and the related SI species *L. vulgaris* (Figure 1). Hybrids were viable and SC, which could provide opportunities to understand S-locus mechanisms as well as consequences of the loss of SI. For example in *Leavenworthia alabamica*, Busch et al. (2011) used a combination of S-locus sequencing and hybrid crosses to show that SC evolution was due to S-linked mutations, but that there were two origins of selfing in this species, with only one showing signs of a severe bottleneck. Similarly, crosses between the SC species *Capsella rubella*

and its SI sister species *C. grandiflora* revealed that SC is caused by a single-dominant mutation suppressing the pollen component of SI (Slotte et al. 2012), and Mable and colleagues (2017) recently used S-locus sequencing techniques to understand the history of divergence in selfing vs. outcrossing populations of North American *Arabidopsis lyrata*. Additionally, hybrids can help to determine how SC alleles, once evolved in one species, might affect the mating system and progeny fitness in a related species following introgression due to hybridization.


**Figure 1.** (a) Plant phenotypic differences between *Linaria cavanillesii* and the hybrid between *Linaria cavanillesii* and *Linaria vulgaris*. (b) Leaves of *Linaria cavanillesii* and (c) leaves of the hybrid (photographs by the author).

### *Leakiness in SI systems*

Leakiness in SI is common in natural plant populations (e.g. Brennan et al. 2005; Crawford et al. 2010; Zhang et al. 2014). For instance, in the Asteraceae family, the study of 571 species revealed that 10% of species show evidence of leaky SI (Ferrer and Good-Avila 2007). However, I still found particularly interesting that some populations of *L. cavanillesii* seem completely SI whereas other show a higher proportion of leakiness. Why should this be the case? It is intriguing that, in the greenhouse some SI plants became leaky in their SI after some time (for instance, plants used as SI in experiments were later seen to produce fruits autonomously), but only in some populations. It would be valuable to determine the impact of leaky SI on populations as well as on the transition toward SC. Is leakiness also transmitted to the progeny, for example? Delayed selfing has been observed in some SC species and seems to show a "best of both worlds" strategy, because it does not reduce outcrossing opportunities but still provides reproductive assurance. Could temporal variation in leakiness observed

in a perennial species function in a similar fashion? What would be the genetic mechanisms explaining this, and why would SI then be maintained in these populations?

*The demographic and evolutionary implications of seed herbivory*

Finally, predation or parasite pressure has been shown to influence mating-system evolution in a way that might conceivably prevent a shift to complete selfing (Agrawal and Lively 2001; reviewed in Campbell 2014; Carr and Eubanks 2014). Specifically, natural enemies have been proposed as a potential force maintaining mixed mating in natural populations, mainly because inbreeding can affect the dynamic between plants and herbivores, which could in return influence the strength of inbreeding depression (Steets et al. 2007). In the field, *L. cavanillesii* populations were strongly impacted by a seed-feeding weevil in the *Rhinusa* family (Curculionidae; Figure 2), which greatly reduced the reproductive success of plants (Appendix 3). I would find it particularly interesting to study in greater detail interactions between the weevils and plants of different mating systems in *L. cavanillesii*. Specifically, I argue previously that pollinators may maintain mixed mating in the SC population, and I wonder how much predation might interact as well. After the molecular identification of the weevil species, Nathalie Jollien (a former master student) and I investigated the behaviour of weevils in the field. We found that their occurrence on a specific plant was related to the flower production of this plant, that female weevils did not discriminate pollinated flowers from virgin flowers for oviposition, and that SI populations suffered from a significantly higher predation rate compared to the SC population, which may suggest a different impact on SI vs. SC plants, and thus on mating-system dynamics. However, we expect this dynamic plant-predator interaction to evolve rapidly, and we do not know how predators would behave in the presence of SI vs. SC plants. This, too, would be worth investigating in greater detail.

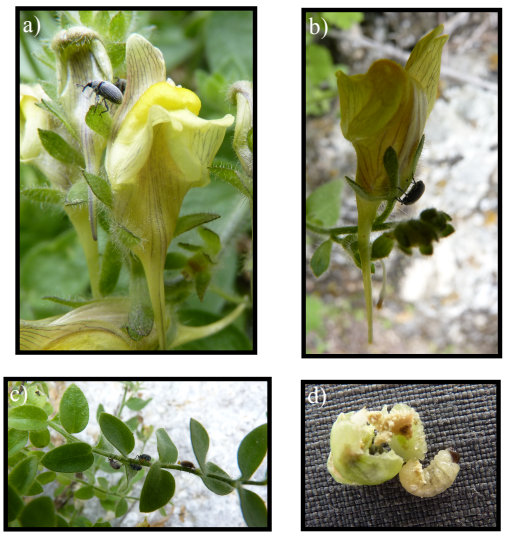



**Figure 1.** Illustrations of the interactions between the plant *Linaria cavanillesii* and the seed-feeder *Rhinusa canescens*. (a) adult visiting an inflorescence to feed on pollen and floral tissues; (b) female piercing the ovary wall to lay eggs in floral ovaries; (c) adults emerging from overwintering, feeding on leaf buds, young leaves and shoot tips and (d) *Rhinusa* larvae developing and eating seeds inside fruits

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# APPENDIX 1. Glossary

**Angiosperms**: also called flowering plants, they are the most diverse group of plants.

**Autonomous selfing:** automatic self-fertilization resulting from the physical contact of anthers with stigmas, without the aid of a pollen vector.

**Competing selfing**: self- and outcross-pollen competing for fertilization on the same stigma of a given flower.

**Delayed selfing:** one case of self-fertilization when the flower self-fertilize only when outcrossing opportunities are over.

**Facilitated selfing**: self-fertilization resulting from an external vector (e.g. pollinators).

**Fitness (Darwinian)**: a relative measure indicating the ability of an individual to survive, reproduce and pass genes to the next generations. It is usually estimated by estimating reproductive components (performance of male and female gametes) and survival.

**Flower display:** number of open flowers on a plant and how they are arranged within and among inflorescences.

**Geitonogamous selfing**: self-fertilization resulting from pollen transferred from one flower to another on the same plant.

**Herkogamy:** Within flower patial separation of anthers and stigma.

**Hermaphrodite**: individuals containing male and female reproductive functions on the same organ. In plants, it represents flowers bearing pistil and stamen (90 % of angiosperm).

**Heterosis:** higher fitness of progeny resulting from between-population crosses vs. within-population crosses.

**Inbreeding depression**: lower fitness of selfed vs. outcrossed offspring, resulting mainly from the expression of deleterious recessive alleles.

**Leaky self-incompatibility**: sporadic production of a low proportion of seeds after self-fertilization, but which result in a reduced seed set compared to outcrossing as opposed to fully self-compatible plant.

**Mating system**: who mates with whom and how often. Here we mainly refered to mating systems as the proportion of seeds that are resulting either from self-fertilization or outcrossing.

**Mixed mating**: fertilization of flowers resulting from a mixture of selfing and outcrossing (it is commonly accepted that plants presenting a selfing rate between 0.2 and 0.8 are classified as mixed maters ; 42 % of flowering plant)

**Outcrossing**: fertilization of one plant by pollen coming from another genetically distinct plant. In most angiosperms, pollinators are necessary to outcross. (contrary: selfing)

**Outcrossing rate**: see *selfing rate.*

**Perennial plant:** plant that is active throughout the year or survives for more than two growing seasons, as opposed to annual or biannual plant.

**Pollen discounting:** reduced potential for outcross siring success because pollen is used for self-fertilization first.

**Pollen limitation**: the reduction in seed production du to the limited amount/quality of pollen available.

**Prior selfing**: selfing occurring before opportunities for outcrossing.

**Pseudo-self-incompatibility (PSI):** see *leaky SI*.

**Purging**: reduced frequency of deleterious mutations resulting from selfing, resulting in a lower mutational load.

**Reproductive assurance:** Advantage of self-fertilizing individuals that can mate in the absence of mates or pollinators.

**Seed discounting:** loss of outcrossing opportunities through seeds due to self-fertilization of ovules.

**Self-compatibility (SC)**: ability to produce seed via self-fertilization (it is usually resulting from the breakdown of genetic mechanism preventing self-fertilization).

**Selfing**: self-fertilization (fertilization of an ovule with pollen grain from the same plant).

**Self-incompatibility (SI)**: genetic mechanism preventing self-fertilization: when a pollen grain reaches a stigma of a similar genotype, a cascade of reactions is preventing fertilization (either at the pollen germination or the pollen-tube growth stage depending on species).

**Selfing rate:** proportion of seeds that result from self-fertilization ($0 < s < 1$). The selfing rate is inversely proportional to the outcrossing rate.

**Selfing syndrome**: changes of flower morphological traits usually following the breakdown of SI mechanisms and the increase in selfing rates, e.g. decrease allocation to pollen export function (such as pollen production or anther-stigma separation), or flower attractiveness (flower size, nectar production).

# APPENDIX 2. S-alleles diversity in four SI populations

**Results of cross-pollination within four self-incompatible populations of *Linaria cavanillesii.*** Mother plants are in columns and pollen donor plants in rows. "1" indicate successful crosses (production of a fruit) whereas "0" indicate incompatible crosses. The diagonals indicate self-pollination.

| **BER** | 79 | 80.6 | 90.5 | 95.1 | 96.3 | 209.1 | 302.2 | 303.3 | 305.2 | 306.3 | 307.3 | 308.1 | 309.4 | 310.1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 79 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| 80.6 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| 90.5 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | NA |
| 95.1 | 1 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| 96.3 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | NA |
| 209.1 | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | NA |
| 302.2 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | NA | 1 | 0 | NA |
| 303.3 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | NA |
| 305.2 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |
| 306.3 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | NA |
| 307.3 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | NA |
| 308.1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 0 | NA |
| 309.4 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 |
| 310.1 | NA | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | NA | 0 |

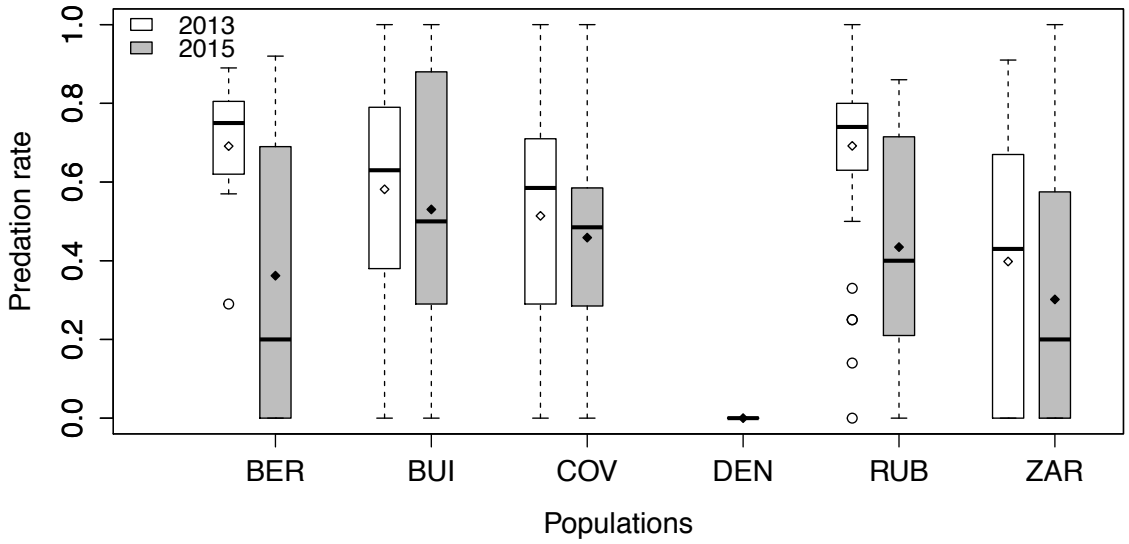
| **BUI** | 40.1 | 53.2 | 69.3 | 312.1 | 313.2 | 314.3 | 316.1 | 320 | 329.1 | 339.1 | 341 | 346.6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 40.1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 53.2 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |
| 69.3 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| 312.1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| 313.2 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 314.3 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| 316.1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 |
| 320 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 |
| 329.1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 |
| 339.1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| 341 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |  | 1 |
| 346.6 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |

| DEN | 1.3 | 2.1 | 3.4 | 7.2 | 14.1 | 16.1 | 25.3 | 30.1 | 31.5 | 42.3 | 44.1 | 240.2 | 248.3 | 269.1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.3 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2.1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 |
| 3.4 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 7.2 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 14.1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 16.1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 25.3 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 30.1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 31.5 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 |
| 42.3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 |
| 44.1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| 240.2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 248.3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| 269.1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 |

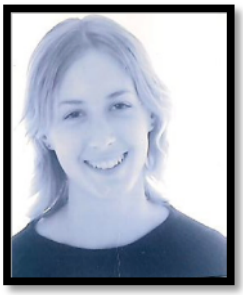
| ZAR | 51.2 | 56.2 | 57.1 | 58.1 | 59.1 | 60.5 | 61.6 | 65.1 | 68.1 | 84.1 | 87.3 | 93.3 | 94.1 | 95.1 | 193 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 51.2 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 56.2 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 57.1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| 58.1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 59.1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 60.5 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 61.6 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 65.1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 68.1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| 84.1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 87.3 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| 93.3 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |
| 94.1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 |
| 95.1 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 1 |
| 193 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 0 |

# APPENDIX 3. Predation rate in six populations of *L. cavanillesii*

**Estimation of predation rate in six natural populations of *Linaria cavanillesii,* in both 2013 (white) and 2015 (grey).** In the boxplots, the middle lines represent median, boxed represent first and third quartiles, lower and upper bars represent the minimum and the maximum, and points represent outliers (i.e., points above 1.5 sd). Diamond symbols represent means for each population.



# VOILLEMOT MARIE



Born April 20th, 1988 in Cahors, France

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## Education

| | |
|---|---|
| **2012-2017** | **PhD thesis** at Lausanne University, Switzerland. *The loss of self-incompatibility and the evolution of mixed mating in the perennial herb* Linaria cavanillesii (supervised by Prof. John R. Pannell) |
| **2010-2012** | **MSc.** option "Behaviour, Evolution and Conservation of the Biodiversity" at Lausanne University, Switzerland**.** Master thesis: *Effect of brood size manipulation and common origin on phenotype and telomere lengths in nestling collared flycatchers,* Ficedula albicollis (supervised by Prof. Pierre Bize) |
| **2009** | **Training with the Tropical Biology Association** in Kirindy forest, Madagascar. Research project: *The impact of distance on the level of between colony aggression in the ant* Aphaenogaster swammerdami *in Kirindy Forest, Madagascar* (supervised by Mary Price) |
| **2008-2009** | **Licence 3 Erasmus** in the Ecology Department of Lund University, Sweden |
| **2006-2008** | **Licence 1 and Licence 2** option "Biology of organisms, populations and ecosystems" at Paul-Sabatier University Toulouse III, France |

## Conferences

| | |
|---|---|
| **2017** | **50th Population Genetics Meeting** (PopGroup), Cambridge (UK). Talk entitled: *Can pollen competition explain the invasion of self-compatibility in mixed populations?* |
| **2016** | **Annual Swiss Conference on Ecology, Evolution, Systematics, Biogeography and Conservation** (Biology16), Lausanne (CH). Talk entitled: *Rapid spread of self-compatibility in different pollinators services* |
| **2015** | **49th Population Genetics Meeting** (PopGroup), Edinburgh (UK). Talk entitled: *Rapid spread of self-compatibility in different pollinators services* |
| | **15th European Society for Evolutionary Biology** (ESEB), Lausanne (CH). Poster presentation: *Rapid spread of self-compatibility in different pollinators services* |
| | **Symposium of Ecology and Evolution for Doctoral Students** (SeeDS), Bern (CH). 3 minutes talk presentation about the thesis. |

| | |
|---|---|
| **2014** | **Doctoriales**, Lausanne (CH). Poster presentation: *Ecological and genetic implications of the loss of self-incompatibility in the toadflax* Linaria cavanillesii |
| | **British Ecological Society** (BES) **and Societé Francaise d'Ecologie** (SFE), Lille (FR). Poster presentation: *Ecological and genetic implications of the loss of self-incompatibility in the toadflax* Linaria cavanillesii |
| **2013** | **NCCR Plant Survival Final Conference**, Neuchâtel (CH). Poster presentation: *Loss of self-incompatibility in the toadfloax* Linaria cavanillesii*: causes and consequences?* |
| **2012** | **Symposium of Ecology and Evolution for Doctoral Students (SeeDS),** Bern (CH) |
| | **Scandinavian Association for Pollination Ecologists** (SCAPE), Skaerhalden Sjobuer (SE). Poster presentation: *Loss of self-incompatibility in the* toadfloax Linaria cavanillesii*: causes and consequences?* |

## Skills

| | |
|---|---|
| **Languages** | French (mother tongue), English (current), Spanish (2000-2006, 2011-2014) |
| **Computer** | Microsoft Word, Power Point, Excel, R, Colony, DYABC, GeneMapper |
| **Laboratory work** | General molecular biology (DNA extraction, PCR, qPCR, microsatellites) |

## Teaching experiences

| | |
|---|---|
| **2015** | Supervision of Samuele Testa, MSc student, University of Lausanne, Switzerland. Master thesis*: Can pollen competition explain differential siring success between mating types of the plant* Linaria cavanillesii*?* |
| **2013** | Supervision of Nathalie Jollien, MSc student, University of Lausanne, Switzerland. Master thesis: *How does variation in self-compatibility of an endangered spanish toadflax relate to levels of seed predation by weevils?* |
| **2017** | Assistant for *Evolution and biogeography of semi-arid and island floras* (John R. Pannell in charge), MSc students, Tenerife, Spain |
| **2016-2017** | Organizer in the public lab *Eprouvette*, University of Lausanne, Switzerland |
| **2013-2017** | Assistant for *Botany* (John R. Pannell in charge), 1st year students, University of Lausanne, Switzerland |
| **2015-2016** | Organizer for the event *Mystères de l'UniL,* group *Trophic interactions* (2015; Philippe Christe in charge), and *Sexuality: what plants can teach us!* (2016; John R. Pannell in charge), University of Lausanne, Switzerland |
| **2013-2016** | Assistant for *Data analysis* (Frédéric Schütz in charge), 2d year students, University of Lausanne, Switzerland |

**2014-2015** Assistant *An introduction to R* (Jérôme Goudet in charge), PhD students, University of Lausanne, Switzerland

**2013-2014** Assistant for *Populations biology* (Nicolas Perrin in charge), 3rd year students, University of Lausanne, Switzerland

**2012** Assistant for *Molecular genetics* (Tania Wyss in charge), MSc students, University of Lausanne, Switzerland

## Scientific publications

**Voillemot M.**, **Rougemont Q., Roux C., Pannell J.R.** (in prep.) The divergence history of the perennial plant *Linaria cavanillesii* confirms a recent loss of self-incompatibility

**Voillemot M., Encinas-Viso F., Pannell J.R.** (in prep.). Rapid transition to self-compatibility in experimental populations of the self-incompatible herb *Linaria cavanillesii*

**Voillemot M., Testa S., Pannell J.R.** (in prep.). Pollen competitive ability promotes the invasion of self-compatibility in mixed populations of a perennial plant

**Voillemot M., Pannell J.R.** (under review in *Heredity*). Inbreeding depression is high in a self-incompatible perennial herb population but absent in a self-compatible population showing mixed mating

**Pannell J.R.**, **Voillemot M.**, 2017. Evolution and ecology of plant mating systems**.** In: eLS. John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0021909.pub2.

**Voillemot M.**, **Pannell J.R.** 2016. Maintenance of mixed mating after the loss of self-incompatibility in a long-lived perennial herb. *Annals of botany*, 119 (1) pp. 177-190

**Pannell J.R.**, **Voillemot M.**, 2015. Plant Mating Systems: Female Sterility in the Driver's Seat. *Current Biology*, 25 (12) pp. R511-R514.

**Voillemot M.**, **Hine K.**, **Zahn S.**, **Criscuolo F.**, **Gustafsson L.**, **Doligez B.**, **Bize P.**, 2012. Effects of brood size manipulation and common origin on phenotype and telomere length in nestling collared flycatchers. *BMC Ecology*, 12 (1) 17

**Waser, N. M., Price M. V., and the TBA Kirindy Pollination Consortium**. 2010. A preliminary early-season flower-visitation web for the Kirindy forest, Madagascar. *Journal of Pollination Ecology*. 2 (1) pp. 1-6